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In Vitro Induction of Apoptosis in Rat Hepatocytes by Cyclosporine A

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ABSTRACT

In rat hepatocytes and isolated liver mitochondrial fractions, CsA is often used as a specific inhibitor of mitochondrial Ca^{2+} release and as a specific blocker of mitochondrial membrane potential and permeability transition (MPT), which are all processes involved in the inhibition of apoptosis. However, neither inhibition nor induction of apoptosis by CsA has yet been described in the rat hepatocyte primary culture during incubation for 4 and 20 hours. It was the purpose of the present study to examine by means of morphological and biochemical criteria the effects of CsA on apoptosis, and to characterize the underlying mechanisms.

Rat hepatocytes were cultured for 4 or 20 hours with CsA at concentrations of 0, 10, 25 and 50 µM. Chromatin condensation and fragmentation, DNA fragmentation (TUNEL), membrane phosphatidylserine distribution (Annexin V), caspase-1, -3 and -6 activity, mitochondrial membrane potential (Rhodamine 123), and cytochrome c release into the cytosol were investigated.

Four hours after CsA treatment, chromatin condensation and fragmentation, and the number of TUNEL- and Annexin V-positive cells increased dose dependently

without any observable enzyme leakage, which indicated the integrity of the outer cell membrane. After 20 hours of CsA incubation apoptosis parameters were further increased and were accompanied by the increased activity of the cysteine protease, caspase-3 (CPP 32), and slightly increased caspase-6 (Mch 2), but not caspase-1 (ICE). The specific caspase-3 inhibitor, Ac-DEVD-CHO, inhibited caspase-3 activation and attenuated CsA-induced apoptosis and LDH leakage. The caspase-6 inhibitor, Ac-VEID-CHO, only marginally inhibited CsA-induced apoptosis. Decreased mitochondrial membrane potential and cytochrome c release went in parallel with ultrastructural mitochondrial changes and might be regarded as early events which trigger the apoptosis cascade. Transmission electron microscopy (TEM) confirmed an increase in the number of necrotic cells after 20 hours, but not after 4 hours, compared with controls.

Key words: Apoptosis.- Ciclosporine A.- Mithochondrial membrane potencial.- Caspases.- DNA fragmentation.

RESUMEN

Inducción de Apoptosis in vitro en hepatocitos de rata por ciclosporina A

La cefalosporina A (CsA) se utiliza frecuentemente en hepatocitos de rata y fracciones mitocondriales hepáticas aisladas como inhibidor específico de la liberación de Ca2+ y como bloqueador selectivo de la permeabilidad y del potencial de membrana mitocondriales, procesos implicados en la inhibición de la apoptosis. Sin embargo, hasta ahora no se ha descrito ni la inhibición ni la inducción de apoptosis por CsA en cultivos primarios de hepatocitos de rata tras su incubación por un periodo de 4 a 20 horas. El propósito de este estudio ha sido examinar con criterios morfológicos y bioquímicos los efectos de la CsA sobre la apoptosis y esclarecer las características de los mecanismos subyacentes.

Los hepatocitos de rata se cultivaron durante 4-20 h. con CsA a concentraciones de 0, 10, 25 y 50 μM. Se investigaron los fenómenos de condensación y fragmentación de la cromatina, fragmentación de ADN (TUNEL), distribución de fostatidilcolina en la membrana (Anexina V), así como la actividades enzimática de caspasas 1, -3 y –6, el potencial de membrana mitocondrial (Rhodamina 123) y la liberación de citocromo C en el citosol.

Tras cuatro horas de incubación con CsA, la condensación y fragmentación de la cromatina y el número de células TUNEL y Anexina V positivas aumentaron en función de la dosis sin que se observara pérdida enzimática, lo que indica la integridad de la membrana celular externa. Después de 20 horas de incubación con CsA, experimentaron un mayor incremento acompañado del aumento de las actividad de cistein-proteasa, caspasa-3 (CPP32) y de un ligero incremento de caspasa-6 (Mch 2), pero no de caspasa-1 (ICE). El inhibidor específico de caspasa-3, Ac-DEVD-CHO,

inhibió la activación de caspasa-3 y atenuó la apoptosis inducida por CsA y la pérdida de LDH. El inhibidor de caspasa-6, Ac-VEID-CHO, únicamente inhibió la apoptosis inducida por CsA. El descenso de potencial de membrana mitocondrial y la liberación de citocromo C fueron paralelos a los cambios de ultraestructuras mitocondriales y pudieran considerarse reacciones tempranas que desencadenan la cascada de fenómenos apoptóticos. La microscopia de transmisión electrónica (TEM) confirmó el incremento del número de células necróticas al cabo de 20 horas, pero no tras 4 horas de incubación, en comparación con los controles.

Palabras clave: Apoptosis.-. Ciclosporina A.- Potencial de membrana mitocondrial.- Caspasas.- Fragmentación de ADN.-

INTRODUCTION

Over the last 20 years, the immunosuppressive drug, CsA, has revolutionized organ transplantation by preventing graft rejection of different organs, and has been successfully used in the treatment of autoimmune disease. In cancer therapy, CsA-related compounds are successfully used in the reversion of multidrug resistance by inhibiting the p-gp 170-kDa protein (Advani *et al.* 1999). Its successful clinical application is accompanied, however, by side effects in the liver (Kahan 1993, Rush 1991). Clinically, CsA-induced hepatic effects are mainly of a cholestatic nature, which manifests itself by elevated serum bile-acid levels (Schade *et al.* 1983), together with hyperbilirubinemia (Soresi *et al.* 1995). In rat hepatocytes, CsA inhibited the uptake and secretion of bile acids, and bile flow in the isolated perfused rat liver (Wolf *et al.* 1998, Kiefer *et al.* 1997). In addition to cholestasis, an increase in serum aminotransferase activity has been observed in some clinical studies with CsA, but this has been found more frequently after treatment with its O-hydroxyethyl-D-$(Ser)^8$-cyclosporine derivative, SDZ IMM125 (Wolf *et al.* 1998). In rats, serum aminotransferase activity increased only after treatment at very high dosages (Donatsch *et al.* 1992). In hepatocyte primary cultures and in the isolated perfused liver, CsA caused the release of lactate dehydrogenase, which correlated very well with that of serum transaminases (Wolf *et al.* 1998, Wolf *et al.* 1997). The leakage of liver-specific aminotransferases into the serum might be regarded as the result

of cellular membrane damage and, hence, necrosis. However, using histopathological criteria, neither apoptosis nor necrosis has been found in man after CsA treatment.

In vivo, CsA induced apoptosis in tubular and intestinal cells of rats(Thomas *et al.* 1998), and in mouse thymocytes (Saiagh *et al.* 1994). In vitro, depending on the cellular system, CsA-induced apoptosis and inhibition of apoptosis has been observed. For example, CsA acts as an inhibitor of apoptosis in T and B lymphocytes (Ito *et al.* 1998) and in neuronal cells (Kruman *et al.* 1999). Induction of programmed cell death has been observed in thymocytes (Huss *et al.* 1995), in glioma cells (Mosieniak *et al.* 1997), and in renal proximal tubular cells (Healy *et al.* 1998).

In rat hepatocyte primary cultures, we recently showed that CsA causes oxidative stress, and that CsA cytotoxicity can be inhibited by antioxidants (Wolf and Broadhurst 1992, Wolf and Donatsch 1990, Wolf *et al.* 1994). The enhanced uptake of Ca^{2+} into liposomal vesicles (Wolf *et al.* 1997) and an increase in extracellular Ca^{2+} (Ellouk-Achard *et al.* 1997) have been demonstrated after treatment of rat hepatocytes with CsA. In the current literature, oxidative stress and increased intracellular Ca^{2+} concentrations have been described as inducers of apoptosis (Buttke and Sandstrom 1994), which suggests the potential role of CsA as an inducer of apoptosis. In contrast, in the current literature, CsA was often used as a specific inhibitor of mitochondrial Ca^{2+} release and of mitochondrial membrane potential, and as a blocker of the MPT in rat hepatocytes (Lemasters *et al.* 1998, Qian *et al.* 1997), all three are processes involved in the inhibition of apoptosis.

To our knowledge, neither inhibition nor induction of apoptosis by CsA has been described in the liver after in vivo treatment. It was recently shown that CsA induced apoptosis in rat hepatocyte cultures treated for 48 hours in the presence of epidermal growth factor (EGF), with the DNA laddering method applied as an indicator of apoptosis (Roman *et al.* 1998).

In order to obtain a better understanding of the nature and mechanisms underlying the hepatic side effects of CsA, the present

investigation aimed to examine CsA action with relation to apoptosis and necrosis by means of morphological and biochemical tools under conditions in which specific hepatocellular functions are preserved during an incubation time between 4 and 20 hours.

MATERIALS AND METHODS

Animals

Permission for animal studies was obtained from the Veterinäramt Basel-Landschaft, CH-4410 Liestal, and all study protocols were in compliance with the institutional guidelines. Male Wistar rats were obtained from Biological Research Laboratories (CH-4414 Füllinsdorf, Switzerland). They were kept in Macrolon® cages with wood shavings as bedding under optimal hygienic conditions, at a temperature of 22-23 °C, a relative humidity of 50-74 %, and fluorescent light for a 12-hour day/12-hour night cycle. They were given water and rodent pellets ad libitum.

Hepatocyte isolation and cell-culture conditions

Rat hepatocytes (rats 180-220 g) were isolated according to the two-step liver perfusion method (Boelsterli *et al.* 1993). The cells were seeded in 35 mm six-well culture dishes[1] at a density of 0.7×10^6 cells in 2 ml WME[2], or in 60 mm culture dishes[1] at a density of 2×10^6 cells in 5 ml WME. The culture medium contained 10 % fetal calf serum, penicillin (100 U/ml), streptomycin (0.1 mg/ml), insulin (10^{-7} M) and dexamethasone (10^{-7} M). After an attachment period of 2 hours at 37 °C in a 5 % CO_2/95 % air atmosphere, the medium was changed. The test compound was added together with the new medium. CsA[3] was dissolved in DMSO, and this solution was added to the culture medium, resulting in a final concentration of 1 % DMSO in the culture medium. Control plates received the DMSO- containing medium without CsA.

The maximum soluble CsA concentration in culture medium in the presence of BSA, without any observable precipitation, was 50 μM.

Transmission electron microscopy

The cell cultures were fixed with 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 hour or overnight at 4 °C. After postfixation with 1 % OsO_4 in 0.1 M cacodylate buffer, pH 7.4, for 1 hour at 4 °C, the cell cultures were dehydrated in graded ethanol solutions and embedded in Epon according to the method of Pease (Pease 1984).

Ultrathin sections of hepatocyte cultures from at least two selected tissue blocks per well were counterstained with uranyl acetate and lead citrate, and were examined with a Philips CM10 transmission electron microscope.

Alterations in the treated cell cultures were expressed by different scores depending on their degree of intensity. The following scores were used: changes observed in one cell per mm^2 = marginal; changes in two cells per mm^2 = slight; alterations in three to nine cells per mm^2 = moderate; alterations in ten or more cells per mm^2 = marked.

Determination of cytotoxicity

Lactate dehydrogenase (LDH) activity in the culture media was measured spectrophotometrically as an index of plasma membrane damage and loss of membrane integrity (Wedler and Acosta 1994). Enzyme activity was expressed as the percentage of extracellular LDH activity of the total LDH activity on the plates.

Determination of chromatin condensation and degradation

Chromatin condensation and fragmentation were determined by Feulgen staining and using light microscopy to count the percentage of cells containing alterations in the nuclear structure (Lillie and Fullmer 1976).

Hepatocyte samples were treated as follows: after the cell culture medium was removed, the cells were fixed overnight with 4 % formaldehyde in PBS. After hydrolyzing in 5 N HCl for 90 min at room temperature, the cells were rinsed in distilled water and stained for 30 min in Schiff reagent[4]. After this, the hepatocytes were rinsed in 0.05 M $Na_2S_2O_3$ solution for 2 min, washed first in tap water, and then washed in

distilled water, and mounted by Crystal Mount[5]. After staining, hepatocyte nuclei were violet in color.

The following criteria were used: normal nuclei were those in which the chromatin was unaltered and uniformly spread over the whole nucleus. Condensed chromatin was located at the nuclear membrane periphery and appeared in a half-moon form. Fragmented chromatin was identifiable by its scattered, droplike structure, which was located on the area of the original nucleus. The total size of apoptotic nuclei appeared to be smaller and more shrunken when compared with intact cells. For each sample, 1000-1500 nuclei were counted.

Determination of DNA fragmentation

TUNEL assay was performed using the DNA fragmentation kit TdT-FragEL™ [6]. The principle of the assay is based on the fact that terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals, and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides (Gavrieli *et al.* 1992). Biotinylated nucleotides were detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzidine reacts with the labeled sample to generate an insoluble, colored substrate at the site of DNA fragmentation. Non-apoptotic cells do not incorporate significant amounts of labeled nucleotide because they lack an excess of 3'-OH ends. Non-apoptotic cells were counterstained with methyl green. Positive TUNEL staining was indicated by a dark brown DAB signal, while shades of blue-green signified a non reactive cell.

Determination of membrane phosphatidylserine distribution

Phosphatidylserine distribution was detected by labeling the cells with the biotin-conjugate of Annexin V[7] according to the method of Vermes (Vermes *et al.* 1995).

Cells were washed with binding buffer (Hepes/NaOH 10 mM pH 7.4, NaCl 140 mM, $CaCl_2$ 2.5 mM) and incubated for 1 hour with Annexin-biotin 1:20 in binding buffer. After washing in PBS, the cells were fixed with formalin. To detect of Annexin V, cells were washed in PBS and incubated with Streptavidin peroxidase complex[8] for 30 min.

After a further washing, the ABC substrate[9] was added for 5-15 min. Cells were washed and mounted with Crystal Mount. Annexin-positive cells were detected by their brown color.

Determination of caspase-1, -3 and -6 activity

Caspase activity was determined according to the method of Rodriguez (Rodriguez *et al.* 1996).

After incubation, 2×10^6 cells were washed once in ice-cold PBS and lysed in 1 ml buffer A (10 mM Hepes, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and protease inhibitors[10]). After three thaw-freeze cycles, the lysate was centrifuged for 20 min at 13000 g at 4 °C. The supernatant (lysate) was removed and stored at –80 °C until the assay was performed.

Lysates (70 μg protein) were assayed in 0.1 % CHAPS, 100 mM Hepes, 10 % sucrose and 10 mM DTT, pH 7.5, with or without protease inhibitors (100 μM); caspase-1 (Ac-YVAD-CHO), caspase-3 (Ac-DEVD-CHO) or caspase-6 (Ac-VEID-CHO)[11] were added in DMSO. The reaction was started with 20 μM of the substrate for caspase-1 (Ac-YVAD-AMC), caspase-3 (Ac-DEVD-AMC) and caspase-6 (Ac-VEID-AMC), which were labeled with the fluorochrome 7-amino-4-methyl coumarin (AMC)[11], and the reaction was followed for 60 min. Fluorescence was measured at excitation 360 nm and emission 460 nm in a fluorescence plate reader.

Fluorescence intensity was calibrated with standard concentrations of 7-amino-4-methyl coumarin (AMC). Protease activity was calculated from the slope of the recorder trace and expressed as pmol/ mg protein/ min.

The difference between the substrate cleavage activity levels in the presence and absence of selective inhibitors reflected the contribution of either caspase-1, -3 or -6 enzyme activity.

Determination of mitochondrial membrane potential

Mitochondrial membrane potential was determined by the uptake of Rhodamine 123 according to the method of Wu (Wu *et al.* 1990).

After treatment, hepatocytes cultured on 96-well plates[1], were washed in PBS and incubated with 10 µg/ml Rhodamine 123[12] for 30 min at 37 °C. After further washing, the hepatocytes were incubated with WME medium for 30 min. Ethanol/Water 1/1 was used to extract the amount of dye retained by the cells. Fluorescence was measured with a Cytofluor 2300 from Millipore, with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Determination of cytosolic and mitochondrial cytochrome c content

Cytochrome c was determined after subcellular fractionation by the Western blotting technique according to the method of Tang (Tang *et al.* 1998).

After incubation, 2×10^6 cells were washed once in ice-cold PBS and lysed in 1 ml buffer A (10 mM Hepes, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and protease inhibitor. After three thaw-freeze cycles, the lysate was centrifuged for 20 min at 13 000 g at 4 °C. The pellet fraction (mitochondria) was first washed in buffer A containing sucrose and then solubilized in 50 µl TNC buffer (10 mM Tris-acetate, pH 8.0, 0.5 % NP-50, 5 mM $CaCl_2$) The supernatant was re-centrifuged at 100 000 g (4 °C, 1 hour) to generate the cytosol, and then removed and stored at -80 °C.

Fifty µg of the cytosolic and mitochondrial fraction was loaded onto a 15 % SDS- polyacrylamide gel and separated according to Laemmli (Laemmli 1970). After fractionation, the proteins were electroblotted onto a PVDF transfer membrane (0.2 µm)[13]. Immunostaining of cytochrome c was carried out with an anti-cytochrome c monoclonal antibody[14]. For detection, the membrane was washed in PBS and incubated with Streptavidin peroxidase complex for 30 min. After further washing, the ABC substrate was added for 5-15 min. Protein bands (15 kD) were revealed using NBT/BCIP.

Determination of protein concentration

Protein content was determined according to Bradford (Bradford 1976). Bovine serum albumin served as standard.

Statistics

A two-way analysis of variance was performed (group and animal; each of them were regarded as qualitative). If the effect of the animal number was not significant it was omitted. A quantile plot was used to visually judge the normality of the residuals. If the residuals were not normally distributed, we tried to achieve (approximate) normality by transforming the response or by omission of outliners.

A multiple comparison method was applied using the three methods of Turkey (Hayter 1989), Sidak (Sidak 1967) and Dunnett (Dunnett 1964). The Dunnett test compares every treated group with the control group, while the other two methods can be used to compare each group with each other group.

The multiple comparison method delivers an estimation of the difference in the response expected between the two groups compared, the standard error of the response, and a lower and an upper confidence limit for the difference. If the two limits do not include zero, the difference is significantly different from zero, on the level of 5 %. By repeating the method for 1 % and 0.1 %, we could analyze how big the significance was.

The S-Plus software (version 5) was used for the computations; the three methods of Turkey (Hayter 1989), Sidak (Sidak 1967) and Dunnett (Dunnett 1964) were used adaptively, i.e. in every case, the most sensitive method was used.

RESULTS

Cytotoxicity

Primary hepatocytes were incubated with CsA at concentrations of 0, 10, 25 and 50 μM for 4 and 20 hours. LDH release was determined as a parameter of cytotoxicity. CsA treatment resulted in a dose- and time-dependent induction of LDH release. While CsA was not cytotoxic at any concentration after 4 hours, CsA was statistically significantly cytotoxic at 25 and 50 μM after 20 hours (Fig.1)

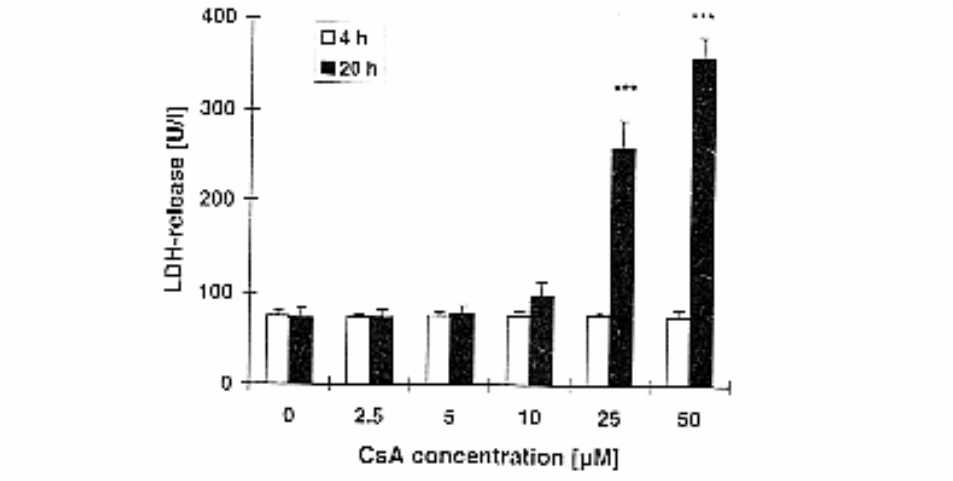

Fig. 1: Effect of CsA on release of lactate dehydrogenase after 4 hours (open square) and 20 hours (closed square). Data are expressed as mean ± SD (n=3). Statistically significant differences versus the control group are expressed as ***P<0.001

Morphology

After 4 and 20 hours of cultivation, untreated control hepatocytes showed a percentage of 1-2 % condensed chromatin. Incubation of the hepatocytes with CsA resulted in a dose-dependent increase in chromatin condensation, with maximum percentages reached at concentrations of 50 µM, i.e. 8.5 times and 14 times above control values after 4 hours and 20 hours, respectively (Fig. 2).

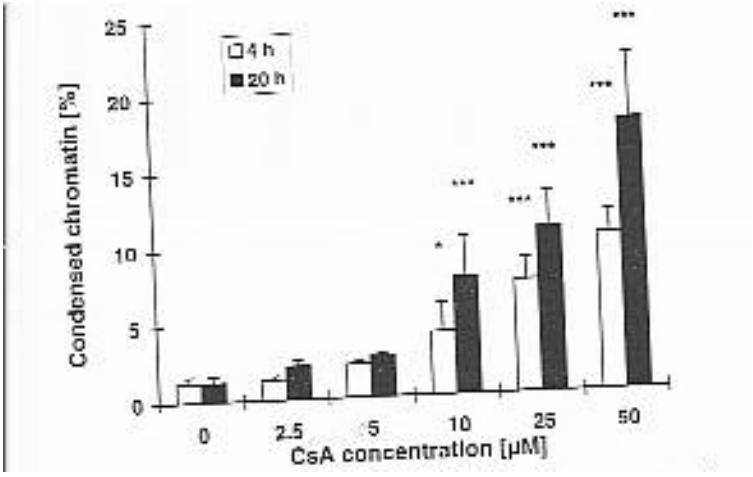

Fig. 2: Effect of CsA on chromatin condensation in hepatocytes after 4 hours (open square) and 20 hours (closed square). Data are expressed as mean ± SD (n=3). Statistically significant differences versus the control group are expressed as *P<0.05 and ***P<0.001.

Chromatin fragmentation in control hepatocytes was 0.1 % after 4 hours and 0.6 % after 20 hours. Under the same conditions, CsA caused a dose-dependent increase in fragmented nuclei. After 20 hours of incubation, 50 µM CsA increased chromatin fragmentation, compared with controls by a factor of 7.4. The effects observed at all concentrations after 20 hours were, on average, about 2-3 times higher than after 4 hours (Fig.3)

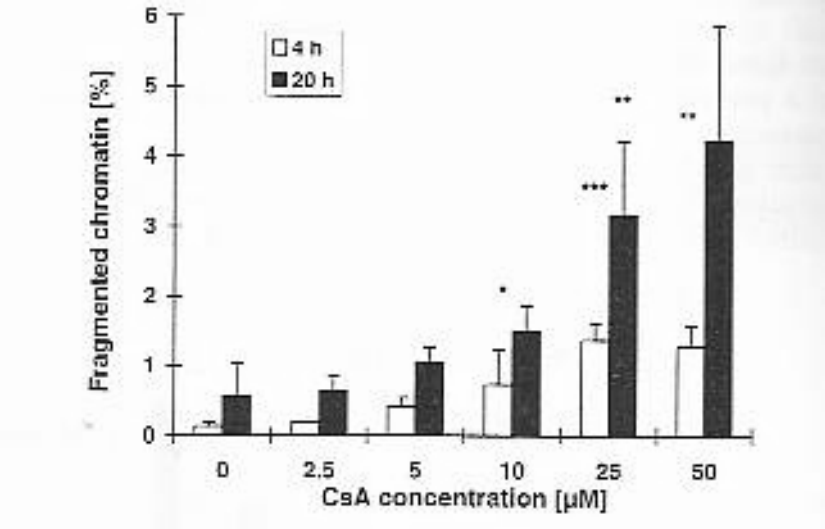

Fig. 3: Effect of CsA on chromatin fragmentation in hepatocytes after 4 hours (open square) and 20 hours (closed square). Data are expressed as mean ± SD (n=3). Statistically significant differences versus the control group are expressed as *P<0.05, **P<0.01 and ***P<0.001.

DNA fragmentation, as determined by the TUNEL assay, showed in a time- and dose-dependent increase after treatment with CsA. At both time points, 50 µM CsA caused a percentage of positive TUNEL

stained

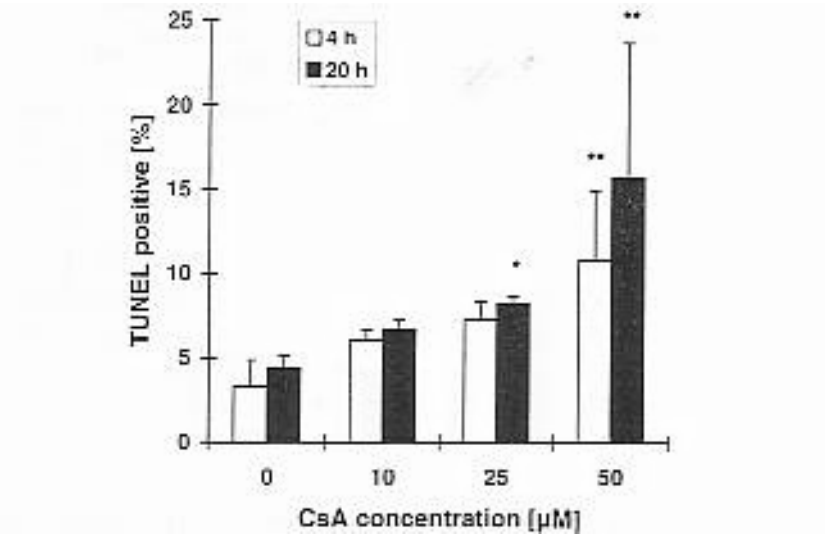

Fig. 4: Effect of CsA on TUNEL-positive hepatocytes after 4 hours (open square) and 20 hours (closed square). Data are expressed as mean ± SD (n=3). Statistically significant differences versus the control group are expressed as *P<0.05 and ***P<0.001.

cells which was about three times higher than that in the control (Fig. 4).

For electron microscopy investigations, hepatocytes were incubated for 4 and 22 hours with 0, 10 and 50 µM CsA. After 4 and 22 hours, hepatocytes from control cultures showed a normal distribution and morphology of all cellular organelles, similar to that observed in hepatocytes in liver tissue (Fig. 5A) (Rhodin 1974, Philips *et al.* 1987, Cheville 1994), with the exception of some cells which had a weakly visible mitochondrial membrane, a few swollen mitochondria, and a marginal dilatation of smooth endoplasmatic reticulum (SER after 22 hours only). A few necrotic cells were observed, and all hepatocytes contained lipid vacuoles and glycogen.

The main ultrastructural changes seen in all treated cultures (4 and 22 hours) were the occurrence of cells with aggregated chromatin in compact masses, condensed cytoplasm with marked crowding of organelles, which was frequently associated with the development of translucent cytoplasmic vacuoles, and cell surface protuberances or blebs. Mitochondria and other organelles maintained their integrity (Fig. 5B-D).

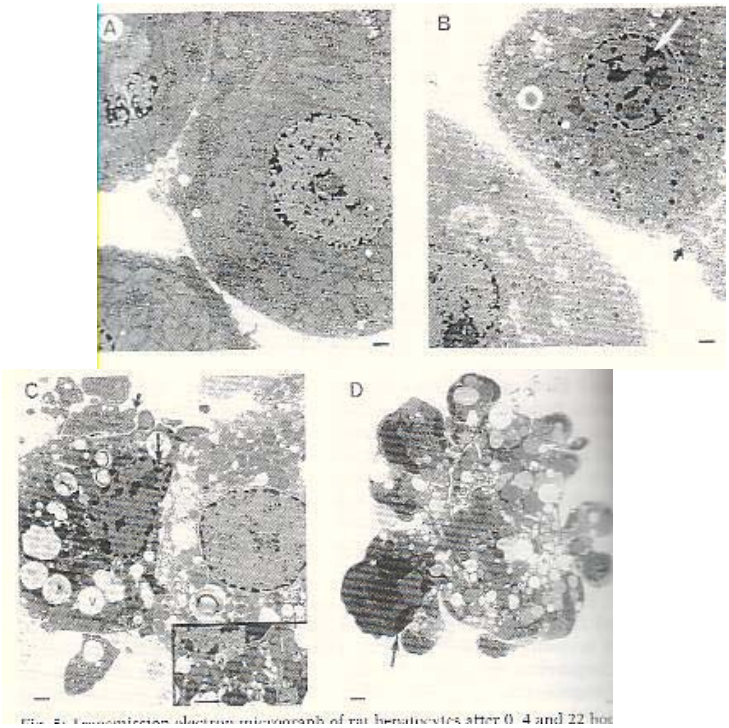

Fig. 5: Transmission electron micrograph of rat hepatocytes after 0, 4 and 22 hours in culture. (A) Control hepatocytes with normal ultrastructure exhibiting distinct mitochondrial membranes and clearly visible cristae. Nucleus appears large and round. Cells contain glycogen. (B-D) Hepatocytes treated with 50 µM CsA for 4 hours (B+D) and 22 hours (C) showing morphological sequence of apoptosis. (B) Note the different ultrastructure of the two cells. Upper right cell has darker mitochondria with clearly visible cristae and membranes *(arrowhead)*, focally dilated endoplasmic reticulum and some blebs *(small arrow)*, and the nucleus is darker and has areas of condensed chromatin *(large arrow)*. (C) Left cell shows condensed cytoplasm with structurally intact mitochondria and other organelles, formation of surface protuberances and cytoplasmic bodies *(small arrow)*, nuclear

shrinkage with invagination of nuclear membrane *(large arrow),* and vacuoles (v). Inset: higher magnification of area from left cell showing mitochondria with intact cristae and membranes *(arrowhead).* (D) Cluster of apoptotic bodies. A variety of organelles is included in the different bodies, and one contains nuclear fragments with condensed chromatin *(large arrow)*. *Bars:* 1.0 μm.

In addition, induction of dilated ER and vacuoles was observed. At 22 hours, all CsA-treated cultures displayed necrotic changes, including swelling of all cytoplasmic compartments, swelling and disappearance of mitochondrial cristae, disintegration of membranes, and accumulation of large, dense granules in the mitochondrial matrix. The necrotic changes were more pronounced in cultures treated with 50 μM CsA (Fig. 6A). There was no significant difference in necrosis in control preparations and CsA-treated preparations after 4 hours of incubation (summary of data in Table 1).

TABLE 1

Synoptic table of ultrastructural findings observed by electron microscopy

			Mitochondrial					
Treatment	SER dilatation	RER dilatation	Membrane weak	Matriz dark	Size/ no.	Apoptotic bodies	Blebs	Necrosis
Control 0h	-	-	-	-	-/-	-	-	-
Control 4h	-	-	(+)	-	S/-	-	-	(+)
CsA 10μM, 4h	(+)	-	(+)	(+)	S/-	(+)	(+)	(+)
CsA 50μM, 4h	(+)	(+)	+	(+)	S,d/ni	(+)	(+)	(+)
Control 22h	(+)	(+)	(+)	-	s/-	-	-	(+)
CsA 10μM, 22h	++	+	+	(+)	s/ni	(+)	(+)	(+)
Csa 50μM, 22h	+++	+	+	+	d/ni	+	+	+

(+) marginal, + slight, ++ moderate, +++ marget, - no alteration/finding, s = swollen,

i = size increased, d = size decreased, ni = number increased

Biochemical markers of apoptosis

Four hours after CsA treatment, Annexin V reaction was increased from 8 % in control preparations to 20 % in hepatocytes treated with CsA (50 µM). The Annexin V reaction showed a similar concentrationdependency as chromatin condensation/fragmentation and DNA fragmentation. Twenty hours after CsA treatment, Annexin V-stained cells increased from 7 % in controls to 30 % (Fig. 7).

After 20 hours of CsA incubation, the activity of the cysteine protease caspase-3 and caspase-6, but not caspase-1, was statistically significantly increased in comparison with controls. CsA treatment at 50 µM resulted in a sevenfold increase in caspase-3 activity; caspase-6 activity increased by 40 %. At the earlier time point (4 hours) no statistically significant increases were seen (Fig. 8).

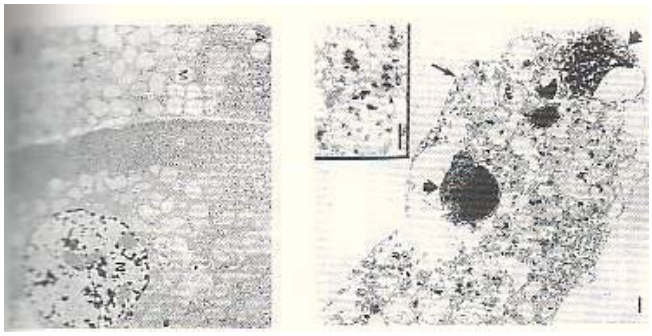

Fig. 6: Transmission electron micrograph of rat hepatocytes. (A) Control culture, 22 hours. Normal cell (upper third), and altered cell showing swelling of all cytoplasm compartments, swelling of mitochondria and disappearance of mitochondrial cristae *(arrowhead)*, and detachment of ribosomes from rough endoplasmic reticulum membranes and vacuolisation *(small arrow)*. Glycogen (g), mitochondria (m), vacuoles (v). (B) Culture treated with 50 µM CsA for 22 hours. Secondary necrosis of apoptotic body: condensed chromatin *(large arrows)* and mitochondrial and cytoplasmic fragments *(small arrow)*. Inset: higher magnification of necrotic

mitochondria characterized by disintegration of membranes and accumulation of large dense granules in the mitochondrial matrix *(arrowhead)*. *Bars:* 1.0 µm.

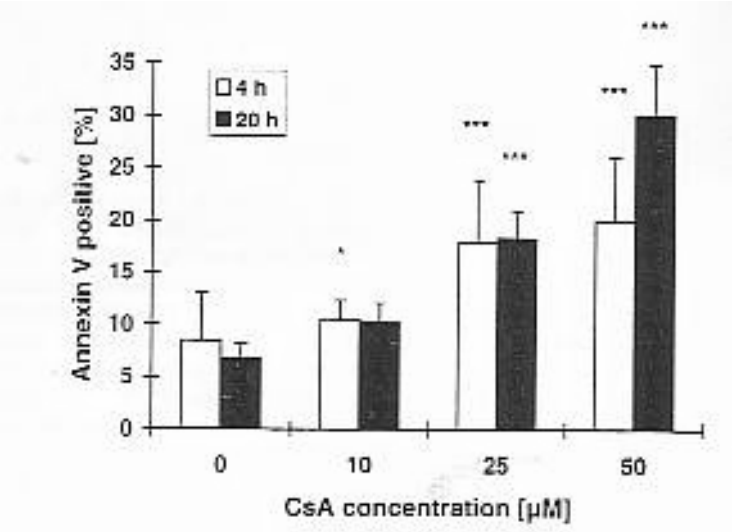

Fig. 7: Effect of CsA on phosphatidylserine distribution after 4 hours (open square) and 20 hours (closed square) of incubation in hepatocyte primary cultures. Data are expressed as mean ± SD (n=3). Statistically significant differences versus the control group are expressed as *P<0.05, **P<0.01 and ***P<0.001.

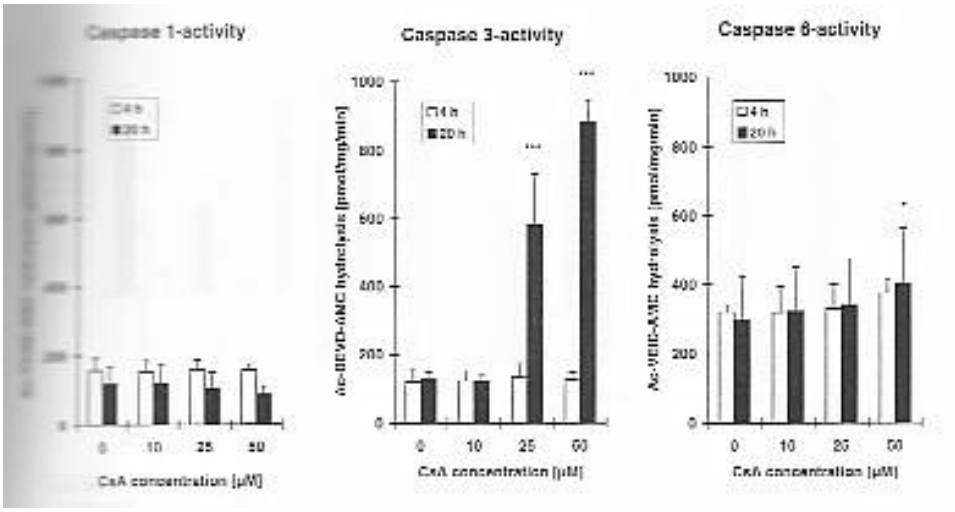

Fig. 8: Effect of CsA on caspase activity after 4 hours (open square) and 20 hours (closed square). Data are expressed as mean ± SD (n=3). Statistically

significant differences versus the control group are expressed as *P<0.05, **P<0.01 and ***P<0.001.

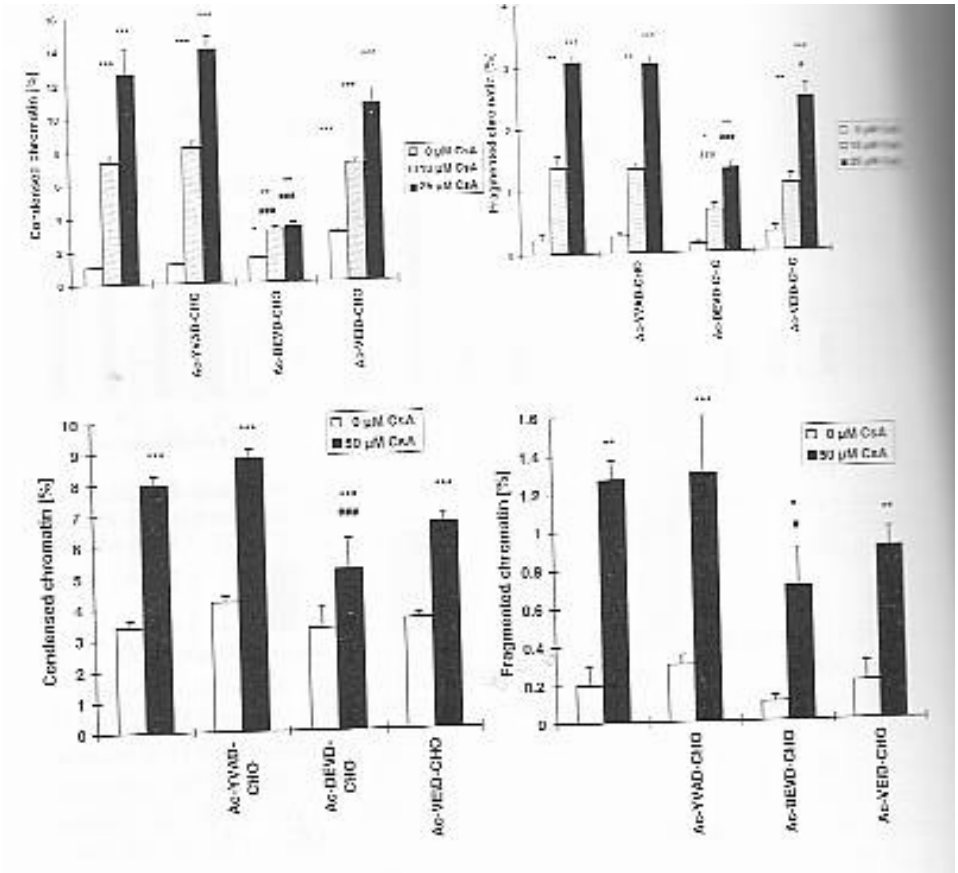

Fig. 9: Effect of caspase inhibitors on CsA-induced chromatin condensation and fragmentation. Hepatocytes were preincubated 1 hour with 100 µM Ac-YVAD-AMC, Ac-DEVD-CHO and Ac-VEID-CHO before adding CsA in combination with the inhibitors.

A: Chromatin condensation after treatment with 0 and 50 µM CsA after 4 hours

B: Chromatin fragmentation after treatment with 0 and 50 µM CsA after 4 hours

C: Chromatin condensation after treatment with 0, 10 and 25 µM CsA after 20 hours

D: Chromatin fragmentation after treatment with 0, 10 and 25 µM CsA after 20 hours

Data are expressed as mean ± SD (n=3). Statistically significant differences compared with 0 µM CsA are expressed as *P<0.05, **P<0.01 and ***P<0.001. Statistically

significant differences compared with the respective CsA group without inhibitor are indicated by $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$.

The specific inhibitors of caspase-1, caspase-3 and caspase-6 were co-incubated at concentrations of 100 µM with 0 and 50 µM CsA for 4 hours and with 0, 10 and 25 µM CsA for 20 hours. After 4 hours, Ac-DEVD-CHO (Nicholson *et al.* 1995) statistically significantly inhibited CsA-induced chromatin condensation and fragmentation to between 50 and 60 % of their initial values (Fig. 9). After 20 hours of incubation chromatin condensation was reduced by the caspase-3 inhibitor by 83 % (10 µM CsA) and 65 % (25 µM CsA). Ac-DEVD-CHO reduced DNA fragmentation by 65 % (10 µM CsA) and 83 % (25 µM CsA). While the caspase-6 inhibitor had only a slight inhibitory effects, on both chromatin condensation and on chromatin fragmentation after 4 hours of incubation, statistically significant 28 % inhibitory effect was found on chromatin fragmentation after 20 hours after coincubation with 50 µM CsA. No effect on chromatin condensation and fragmentation was observed with caspase-1 inhibition.

After 20 hours of incubation, CsA-induced LDH release (50 µM) was reduced by the caspase-3 inhibitor, Ac-DEVD-CHO, to nearly 45 % of leakage values obtained with CsA without the inhibitor. No significant

effect was observed with the caspase-1 or caspase-6 inhibitor (Fig.10)

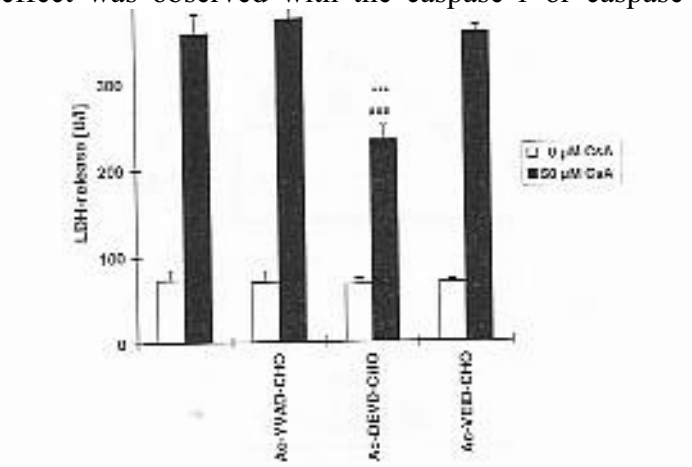

Fig.10: Effect of caspase inhibitors on CsA-induced LDH release. Hepatocytes were preincubated 1 hour with 100 µM Ac-YVAD-AMC, Ac-DEVD-CHO and Ac-VEID-CHO before adding 50 µM CsA together with the inhibitors. LDH release was measured after 4 hours and 20 hours of incubation.

Data are expressed as mean ± SD (n=3). Statistically significant differences compared with 0 µM CsA are expressed as *P<0.05, **P<0.01 and ***P<0.001. Statistically significant differences compared with the 50 µM CsA group are indicated by $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001.

Hepatocyte cultures were incubated at CsA concentrations of 0, 0.1, 0.5, 1, 2.5, 5, 10, 25 and 50 µM for 1, 2, 4 or 20 hours. Mitochondrial membrane potential decreased after 1 hour of CsA incubation by about 50 % of the control at the highest CsA concentration (50 µM). After 20 hours of CsA incubation at 50 µM, Rhodamine 123 uptake decreased maximally by 75 % of control values. The decrease in membrane potential was dose and timedependent (Fig.11).

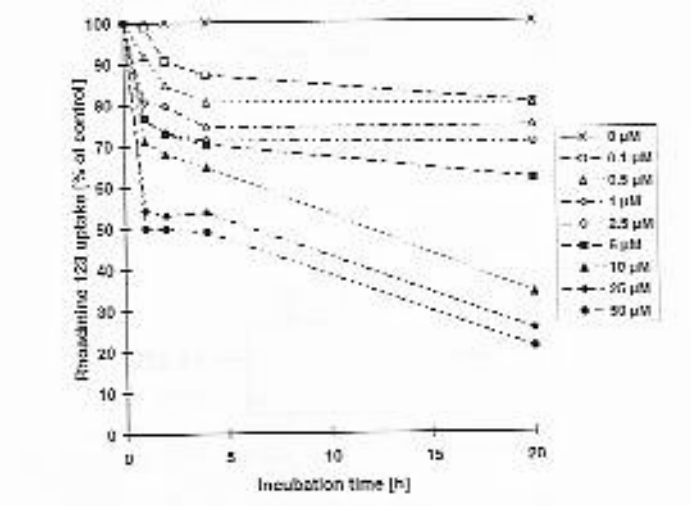

Fig.11: Effect of CsA on the mitochondrial membrane potential after 1, 2, 4 and 20 hours of incubation in hepatocyte primary cultures.

Cytochrome c release from the mitochondria into the cytosol was determined after 4 and 20 hours of CsA incubation. Four hours after CsA treatment (50 µM), there was an approximate twofold increase in cytochrome c in the cytosol, and a decrease in the mitochondria. After 20

hours, this effect was even more pronounced (Fig.12).

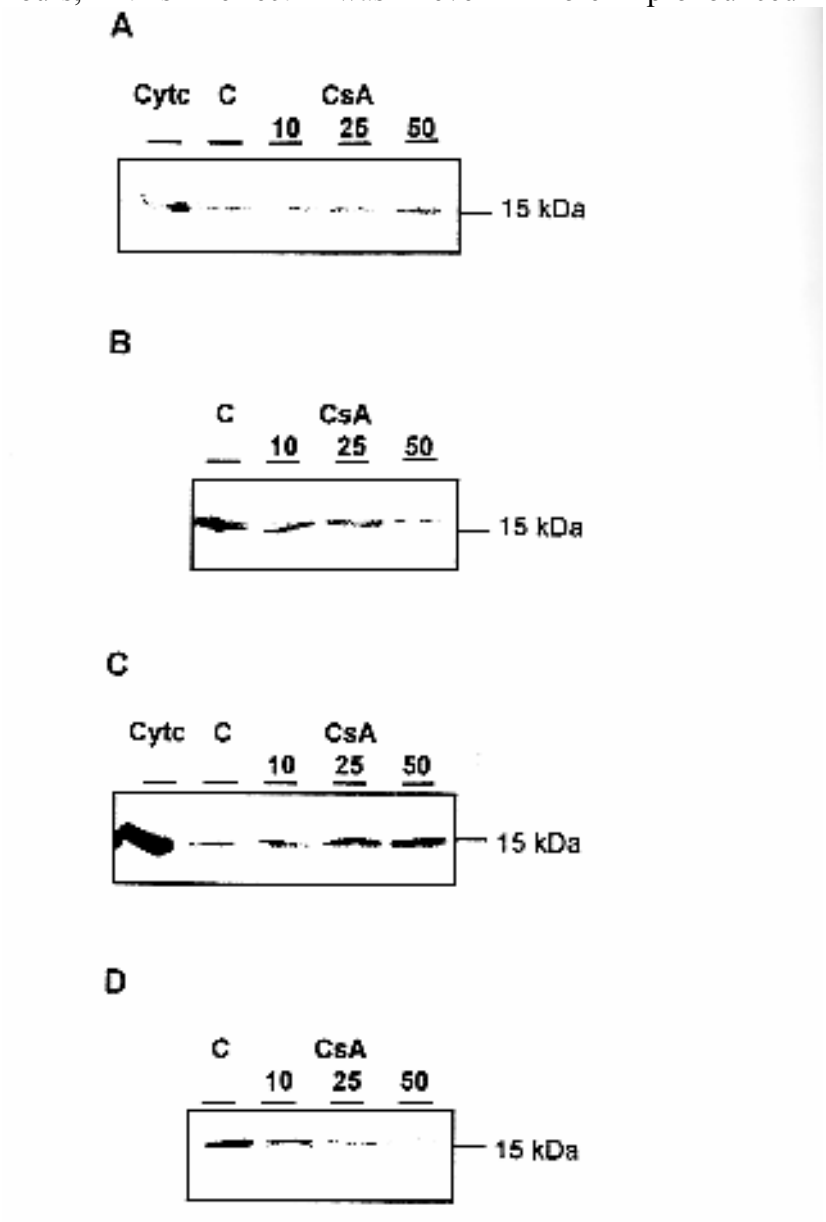

Fig.12: Effect of CsA on cytochrome c release after 4 and 20 hours. Immunoblot of cytosol and mitochondria after 4 hours and 20 hours of treatment.

A: Cytosol after 4 hours, B: Mitochondria after 4 hours; C: Cytosol after 20 hours; D: Mitochondria after 20 hours. C = Control cells; Cells treated with either 10 μM, 25 μM and 50 μM CsA.

DISCUSSION

Cell death induced by a foreign compound may occur from two major mechanisms, necrosis and apoptosis (Fawthrop *et al.* 1991, Wyllie *et al.* 1980). Necrotic cell death can occur from noxious injury, while apoptosis is an endogenous cellular process in which an external signal activates a metabolic pathway that results in cell death. This type of cell death is a common feature in cellular differentiation and other biological processes that regulate cell numbers. In addition, apoptosis is triggered by various xenobiotics, such as antineoplastic agents, or after removal of growth factors. While necrotic cell death results in cell lysis and destruction of the outer plasma membrane, cellular apoptosis is morphologically characterized by cell shrinkage, nuclear pyknosis, chromatin condensation and degradation, blebbing of the plasma membrane, and solubilization of the nuclear matrix (Fawthrop *et al.* 1991, Eanshaw 1995, Miller *et al.* 1993).

In the present study, by applying morphological and biochemical methods and determining apoptosis at different subcellular levels, we have shown, for the first time, that CsA specifically induced apoptosis in primary rat hepatocyte cultures after 4 hours and 20 hours of treatment.

At the nuclear level, we found that CsA induced an increase in chromatin condensation and fragmentation as determined by light microscopy after Feulgen staining and TEM investigation. Feulgen staining is regarded as a specific method for chromatin staining (Lillie and Fullmer 1976), and in our investigations we found that CsA induced apoptosis according to the recognized criteria detailed above in the methodological part of this paper. The light microscopy data were

supported by ultrastructural evaluations using TEM. TEM was used for qualitative assessment of the structural changes and did not permit a full quantitative assessment of the findings. With TEM it was possible to demonstrate that CsA induced apoptosis according to classical criteria and to distinguish the effects observed from necrosis. Apoptosis appeared as typical cell shrinkage accompanied by condensed cytoplasm with focally dilated endoplasmic reticulum, structurally intact mitochondria and other organelles compacted together and maintaining their integrity, formation of cytoplasmic protuberances on the cell surface (blebbing) and cytoplasmic bodies, nuclear shrinkage with invagination of nuclear membrane, and massive deposits of chromatin in compact masses adjoining the nuclear envelope. It was also possible to clearly distinguish necrosis characterized by swelling of all cytoplasmic compartments, swelling and disappearance of mitochondrial cristae, disintegration of membranes and accumulation of large dense granules in the mitochondrial matrix, detachment of ribosomes from rough endoplasmic reticulum membranes, and nuclear swelling with clumping of loosely textured nuclear chromatin (Wyllie *et al.* 1980).

The results obtained from morphological investigations were supported by the specific determination of DNA fragmentation using the TUNEL method. The results showed that CsA specifically causes DNA damage by the generation of 3'-OH end fragments. This method is considered to be specific because of the binding of the terminal deoxynucleotidyl transferase (TdT) to exposed 3'-OH ends of DNA fragments generated in response to apoptotic stimuli (Gavrieli *et al.* 1992). The loss of plasma membrane asymmetry is another feature, and is an early process in apoptosis, preceding apoptotic DNA degradation (Eanshaw 1995, Martin *et al.* 1995).

In the present investigation, membrane phosphatidylserine distribution was used as a sensitive parameter of apoptosis to indicate plasma membrane asymmetry. In normal cells, phosphatidylserine is located exclusively in the inner cytoplasmic surface of the membrane. In apoptotic cells, phosphatidylserine flips within the cell membrane to become located on both surfaces. This is an early event in the apoptotic

process, which is used to detect apoptotic cells before gross morphological changes become visible. The phospholipid-binding protein Annexin V has a high affinity for phosphatidylserine and serves as a very specific marker for apoptotic cells. In the current study, CsA-induced Annexin V reactive cells increased dosedependently, at all time points, in parallel with chromatin changes and DNA degradation.

Annexin V staining, chromatin condensation and fragmentation and the TUNEL assay have also a certain potential to give positive results with necrotic cells (Gold *et al.* 1994, Vermes *et al.* 1995). By means of ultrastructural investigation with TEM we could exclude the possibility of necrosis at 4 hours after CsA treatment at all concentrations. Exclusion of necrosis can also be supported by the determination of LDH activity in the cell culture supernatant, proof of the integrity of the outer cell membrane, a typical criteria for necrotic cell death. The results obtained from the parameters of apoptosis applied after 4 hours can therefore be considered to be highly specific for the induction of apoptosis.

There is no general mechanism for the induction of apoptosis; induced by different mediators, it may occur by different mechanisms. Mitochondria, however, are thought to play a central role in the activation of apoptosis, induced by multiple agents, by a mechanism that involves breakdown of mitochondrial membrane potential and release of cytochrome c and its binding to a multiprotein complex that activates caspase-3 (Pan *et al.* 1998).

Our data strongly suggest that CsA induces apoptosis by decreasing mitochondrial membrane potential, which is followed by MPT as determined by cytochrome c release from the mitochondria. This finding is in conflict with the current literature. CsA has been described by many authors as a very specific inhibitor of mitochondrial membrane potential and permeability transition in isolated mitochondrial fractions and in isolated hepatocytes (Lemasters *et al.* 1998), when incubated for 1 hour at concentrations in the nanomolar range. Under our experimental conditions, we found a rapid loss of mitochondrial membrane potential 1 hour after CsA treatment at 1 μM. After 1 hour of incubation with 50 μM CsA, mitochondrial membrane potential decreased to 50 % of that of control cells. CsA-mediated MPT was observed after 4 hours, and

significantly increased apoptosis was found after 4 hours at concentrations of 10 μM. The difference in our data to those of other investigators might be explained by the different CsA concentrations used and differences in the treatment periods. CsA can serve as a specific inhibitor at low concentrations and after short-term exposure, but not after longer treatment with high concentrations.

CsA-induced MPT can have several consequences. One consequence is the release of apoptosis-inducing factors (AIF), which could be the cause of the increase in phosphatidylserine observed in the outer leaflet of the cytoplasmic membrane, possibly by decreasing flippase activities or inhibiting inactivated phosphatidylserine translocases. Among others, cytochrome c may serve by itself as an AIF, which, once it has been released from the mitochondria, can activate the caspase cascade. Caspases are important effector molecules, which trigger the biochemical events in apoptosis and ultimately execute apoptotic cell death. The caspases are probably the most important effector molecules in the apoptotic process. In general, caspases are present as inactive proenzymes that are activated by specific proteases, in some cases by autocatalysis (Guerrero and Arias 1998). Caspases are organized in enzymatic cascades so that several upstream caspases are able to activate downstream caspases. The presence of a protease cascade has been suggested by the sequential appearance of proteolytic activity cleaving fluorescent YVAD and DEVD substrates (Enari *et al.* 1996). In our study, the participation of caspase-3 in the induction of CsA-induced apoptosis can, at this time, be concluded only indirectly. In our experiments, we observed a dose-dependent increase in caspase-3 activity after an incubation of 20 hours with CsA, but not after 4 hours, although apoptosis has also been clearly shown at this time point. This result principally demonstrates that caspase-3 might be involved in the induction of CsA-induced apoptosis after 20 hours. In comparison to this, the specific caspase-3 blocker, Ac-DEVD-CHO was able to inhibit CsA-induced chromatin condensation and fragmentation already after 4 hours, which indicates that caspase-3 might be involved in the early apoptotic steps. As well as the possible time-specific action of caspase-3, it also seems possible that the activity of caspase-3 is not visible or is hidden after

4 hours in the case of CsA. Under the same conditions, the cyclosporin derivative, IMM 125, an inducer of apoptosis which is twice as strong as CsA, significantly increased caspase-3 activity after 4 hours (paper in preparation). The differences in the CsA-induced, caspase-3 activity dose-response curve and the chromatin condensation/fragmentation dose-response curves might be due to analytical reasons. While the natural substrate of caspase-3 is poly-ADP-ribose-polymerase (PARP) (Thornberry and Lazebnik 1998, Inayat-Hussain *et al.* 1997), in our analytical test system we used an artificial substrate, Ac-DEVD modified 7-amino-4-methyl coumarin (AMC), which could have a different affinity to the enzyme, and thus be less sensitive. In addition, the strong background noise signal in the analytical assay of caspase-3 activity did not allow the sensitive detection of very small increases in comparison with controls.

Caspase-6 activity was also found to be statistically significantly increased by CsA, but these effects were only minor. Its minor role was also demonstrated by the relatively weak effect of the specific inhibitor, which showed an inhibitory effect on chromatin condensation and fragmentation only after 20 hours at a low magnitude of inhibition.

The results obtained by Ac-DEVD-CHO suggest that necrosis might be in close relation to apoptosis. In our experiments, we also found that the caspase-3 inhibitor not only prevented CsA-induced apoptosis, but also LDH release. Since LDH leakage indicates plasma membrane damage, this is a parameter which, under the given conditions, could serve to determine necrosis. Results obtained by TEM would support such an assumption, especially under in vitro conditions. Some apoptotic cells with necrotic properties were seen. Within tissue, apoptotic bodies usually undergo rapid phagocytosis. However, in cell cultures, such bodies can undergo spontaneous degeneration with swelling and membrane rupture (Eanshaw 1995). The necrosis observed in the cultures in the present study could partly be “secondary necrosis” of apoptotic bodies (Eanshaw 1995). It seems possible that transition from apoptosis into necrosis may also occur in vivo.

Our results suggest that the mechanism of CsA-induced apoptosis proceeds from disruption of mitochondrial membrane potential to

destruction of the mitochondrial membrane, resulting in cytochrome c release into the cytosol. This results in an activation of caspases, especially caspase-3, and, to a less extent, caspase-6. As a consequence, DNA is attacked, as observed by condensation and fragmentation of chromatin.

Although Ac-DEVD-CHO and Ac-VEID-CHO inhibited CsA-induced chromatin condensation and fragmentation, the blockage of apoptosis was, in all cases, not complete. In our experiment, we used inhibitor concentrations which were supposed to be specific according to the current literature (Inayat-Hussain *et al.* 1997). Thus, it can be assumed, that the concentrations to achieve full inhibitory action were sufficient to demonstrate the involvement of both of these enzymes. That we did not succeed in total inhibition may indicate that not only caspases are involved in the process of CsA-induced apoptosis, but also other mechanisms.

We cannot exclude that there exist other mechanisms, parallel with the activation of caspases, which result in CsA-induced apoptosis. It is also possible that intracellular calcium plays a role in this complex mechanism. This would be consistent with the hypothesis of Jewell *et al.* (Jewell *et al.* 1982), who postulated that morphological changes in the plasma membrane are associated with disturbances in intracellular calcium homeostasis. It is known that an increase in intracellular calcium concentration may directly cause toxicity. Free calcium can serve as a mediator of cytotoxicity by means of activating catabolic pathways by which important cellular macromolecules such as proteins, lipids and nucleic acids are degraded (Orrenius 1993, Richter and Kass 1991). Activation of Ca^{2+}-dependent endonucleases could lead to the typical apoptotic DNA ladder pattern (Guerrero and Arias 1998). Although inhibition of CsA-mediated Ca^{2+} efflux from mitochondria has been described by Richter *et al.* and several other authors (Richter and Kass 1991, Nicchitta *et al.* 1985, Kehrer *et al.* 1993), there is good evidence that the uptake of extracellular Ca^{2+} is enhanced by CsA in intact hepatocytes (Ellouk-Achard *et al.* 1997), in other cell types, and also in liposomal fractions (Wolf *et al.* 1997, Meyer-Lehnert and Schrier 1989,

Lo Russo *et al.* 1996) Reactive oxygen species (ROS) are also supposed to be inducers of apoptosis in various in vitro cellular systems (Jabs 1999), and in agreement with this, inhibition of apoptotic cell death has been observed with antioxidants (Jabs 1999). Under certain circumstances, ROS might serve to trigger the increase in intracellular Ca^{2+} concentrations, and, by this process, leading to the induction of apoptosis (Orrenius 1993); a mechanism different from that with caspases. We have also recently found that CsA induced the formation of ROS, which makes the assumption of such a mechanism very likely (Wolf *et al.* 1997).

In summary, the present results showed that, in rat hepatocyte primary cultures, CsA specifically induced apoptosis after short-term incubation; this overlapped with necrosis after longer-term incubation. It is likely that the necrosis which occurrs after 20 hours is, partially, a secondary result of apoptosis.

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ABBREVIATIONS

Ac	Acetate
AMC	7-amino-4-methyl coumarin
$[Ca^{2+}]_i$	Intracellular calcium
CHO	Aldehyde
CPP 32	Caspase-3
CsA	Cyclosporine A
DEVD	Asp-Glu-Val-Asp
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EGF	Epidermal growth factor
ICE	Interleukin-1β-converting enzyme, caspase-1
LDH	Lactate dehydrogenase
Mch 2	Caspase-6
MPT	Mitochondrial permeability transition
MW	Molecular weight
NBT/BCIP	Nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate
PARP	Polyadenosylribosepolymerase
PBS	Phosphate buffered saline
RER	Rough endoplasmic reticulum
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SER	Smooth endoplasmatic reticulum

TEM	Transmission electron microscopy
TUNEL	TdT-mediated dUTP-biotin nick end labeling
VEID	Val-Glu-Ile-Asp
WME	William's medium E
YVAD	Tyr-Val-Ala-Asp

Footnotes

[1] Primaria; Falcon, Switzerland

[2] Gibco, BRL Life Technologies AG, Switzerland

[3] Novartis, Switzerland

[4] Merck, Switzerland

[5] Biomeda, USA

[6] Calbiochem, Switzerland

[7] Roche, Switzerland

[8] Elite Kit Vector; Vector, USA

[9] Biomeda, USA

[10] Complete; Roche, Switzerland

[11] Bachem, Bubendorf, Switzerland

[12] Molecular Probes, Netherlands

[13] Bio-Rad, Switzerland

[14] 7H8.2C12; Pharmingen, USA