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The immunosuppressant drug FK506 prevents Fas-induced apoptosis in human hepatocytes

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Abstract

FK506 is a potent immunosuppressive drug used for the prevention of graft rejection in organ transplantation. Experimental and clinical studies have shown correlations between apoptosis and graft rejection, and apoptosis also plays a role in cell death after ischemia–reperfusion injury in the rat liver. Fas-mediated apoptosis is very likely involved in allograft rejection and experimental evidence has shown a decrease of FasR expression in mouse hepatocytes produced by the drugs. On the basis of these findings we have investigated the protective effect of FK506 in comparison with cyclosporine A (CsA) on Fas-induced apoptosis, by analysing the activation of downstream effector caspases in human hepatocytes. Apoptosis was induced by treatment with agonistic antibodies against FasR, which resulted in a significant activation of caspase-3 after 12 h. Prevention of the downstream activation of the caspase cascade and apoptosis was observed when hepatocytes were pre-treated for 3 h with immunosuppressant drugs. A significant reduction (ca. 30–40%) of caspase-3 activation by 5 μM FK506 and CsA was observed. Along with less activation of caspase-3 a decrease of apoptotic DNA fragmentation was found. In addition, FK506 significantly reduced not only caspase-8 but also caspase-9 activation, to a similar extent as CsA, thus suggesting a protective effect at the mitochondrial level of this drug, as has already been reported for CsA. These effects of FK506 help to explain its strong anti-rejection properties and suggest promising benefits of pharmacological preconditioning on ischemia–reperfusion injury following liver transplantation.

Keywords: Cyclosporine A; Caspases-3, -8 and -9; DNA fragmentation; Fas; FK506; Mitochondria

1. Introduction

Apoptosis has long been recognized as an important mechanism of cell death in transplantation-associated liver damage, mainly related to allograft rejection [1]. Both experimental findings in rat liver transplantation [2] and studies in patients with acute and chronic rejection [3–5] show that hepatocyte apoptosis correlates with graft rejection. In addition, some authors have observed that apoptosis might be involved in cell death after ischemia–reperfusion injury in the rat liver [6].

The apoptotic stimuli comprise extrinsic signals (binding of death-inducing ligands to cell surface receptors), and intrinsic signals produced following cellular stress (initiate apoptosis involving the mitochondria). Therefore, the two main pathways of apoptosis include: (a) the cell death receptor pathway, including the FasR is activated by its ligand, FasL, triggers the clustering of the death domain that mediates transduction of the death signal activating the apoptosis program [7] and (b) the mitochondria-initiated pathway that involves release of mitochondrial proteins which commits hepatocytes to apoptosis [8–10]. Fas-

Abbreviations: CHX, cycloheximide; CsA, cyclosporine A; DMSO, dimethylsulfoxide; DUBQ, decylubiquinone; FasR, Fas receptor; FasL, Fas ligand; LDH, lactate dehydrogenase; MNTC, maximal non toxic concentration

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mediated apoptosis very likely takes place as a mechanism of allograft rejection [4]. Hepatocytes are rich in FasR under certain inflammatory conditions [4] and cytotoxic T-lymphocytes express FasL [11]; thus, T-cells could activate hepatocyte apoptosis in allograft rejection. Moreover, recent clinical reports show a significant increase of FasR expression in hepatocytes, percentage of apoptotic hepatocytes and higher levels of FasL in serum of patients with liver allograft rejection than in controls [12].

The macrolide drug FK506 (Tacrolimus) is a potent immunosuppressive agent used for the prevention of graft rejection in organ transplantation. It has been claimed to possess hepatotrophic or hepatoprotective properties to reduce liver injuries of various etiologies, which are independent of the inhibition of T-cell activation, and might contribute to the anti-rejection properties of FK506 [13–15]. A protective anti-apoptotic effect in the liver cells could be one of these actions. Evidence showing a decrease of FasR expression in mouse hepatocytes cultured in presence of FK506 has also been reported [16,17]. FK506 has also been shown to have down-regulatory effects on various cellular and biochemical mechanisms involved in liver ischemia–reperfusion damage [18]. FK506 has, therefore, been proposed as a pharmacological preconditioning agent with promising beneficial actions on ischemia–reperfusion injury following liver transplantation [19], which has been related partly to the prevention of apoptosis both in cultured rat hepatocytes [20] and in patients [21].

These previous findings showing how FK506 prevents apoptosis in liver cells are of great clinical and pharmacological relevance. Demonstration of a direct action of FK506 on human hepatocyte apoptosis could be of interest to help to understand its anti-rejection properties. Therefore, the influence of FK506 on Fas-mediated apoptosis, the activation of downstream effector caspases and DNA fragmentation was investigated in human hepatocytes, and compared with the immunosuppressant CsA, an agent whose protective effect on hepatocyte apoptosis during ischemia–reperfusion injury has already been reported [22,23].

2. Material and methods

2.1. Reagents

FK506 was supplied by Fujisawa (Osaka, Japan). CsA was purchased from Sigma (MO, USA). Collagenase was from Roche (Barcelona, Spain). Ac-DEVD-AMC caspase-3 and Ac-IETD-AFC caspase-8 fluorogenic substrates were from PharMingen (San Diego, CA). Ac-LEHD-AFC caspase-9 fluorogenic substrate and IETD-CHO and LEHD-CHO (caspase-8 and caspase-9 cell permeable inhibitors, respectively) were purchased from Calbiochem (Nottingham, UK). Culture media (Ham's F-12, Lebovitz L-15) and DNase I Amplification Grade were from Gibco BRL (Paisley, UK). Anti-Fas monoclonal antibody CH11 clone was purchased from MBL (Nagoya, Japan). All other chemicals were of analytical grade.

2.2. Isolation and culture of human hepatocytes

Human hepatocytes were obtained from elective liver biopsies (1–5 g) in the course of a therapeutic laparotomy for non-malignant liver disease or extrahepatic disease after receiving informed consent from patients, in conformity with the rules of the Hospital's Ethics Committee [23,24]. None of the patients were habitual consumers of alcohol or other drugs. A total of 13 cell preparations from different liver biopsies were used in the study (Table 1). Hepatocytes were isolated using a two-step perfusion technique and cultured as described [25]. Briefly, the tissue was extensively washed with a Ca^{2+}-free buffer containing 0.5 mM EGTA and in the second step was perfused with the same buffer, without EGTA but with 5 mM $CaCl_2$, and collagenase for 1 h. Cellular viability was assessed by the Trypan blue dye exclusion test. Hepatocytes were seeded on fibronectin-coated plastic dishes (3.5 μg/cm^2) at a density of 8×10^4 viable cells/cm^2 and cultured in Ham's F-12/Williams (1:1) medium supplemented with 2% newborn calf serum, 50 mU/ml penicillin, 50 μg/ml streptomycin, 0.1% bovine serum albumin, 10^{-8} M insulin, 25 μg/ml transferrin, 0.1 μM sodium selenite, 65.5 μM ethanolamine, 7.2 μM linoleic acid, 17.5 mM glucose, 6.14 mM ascorbic acid, and 0.64 mM *N*-omega-nitro-L-arginine methyl ester. The medium was changed 1 h later to remove unattached hepatocytes. By 24 h, the cells were shifted to serum-free hormone-supplemented medium (10 nM dexamethasone and insulin). Treatments started 16–20 h after seeding.

2.3. Preparation of stock solutions for treatment of cultures

Stock solutions of 500 μM FK506 in DMSO, 1 mg/ml cicloheximide in water, and 400 μM CsA in PBS were

Table 1
Characteristics of the biopsies used in the study

Biopsy	Gender	Age	Cell viability (%)
1	Male	73	93
2	Female	47	96
3	Male	78	91
4	Male	22	99
5	Female	39	99
6	Male	59	99
7	Male	60	97
8	Female	47	94
9	Female	70	95
10	Male	77	98
11	Male	49	97
12	Male	28	98
13	Male	26	97

prepared. A 500 ng/μl commercial solution of anti-Fas antibody, which is known to induce active cell death based on Fas-mediated apoptosis [26], was diluted in culture medium. Stock solutions of 400 mM of IETD-CHO and LEHD-CHO (caspase-8 and caspase-9 cell-permeable inhibitors, respectively) were prepared in DMSO and added to hepatocytes at a final concentration of 100 μM. Stock solutions were diluted with culture medium to obtain the appropriate final concentrations. In all cases the concentration of solvent in culture medium did not exceed 0.5% (v/v).

2.4. Cytotoxicity assay

Increasing concentrations of the drug in PBS were added to cultures after medium renewal and cells were incubated for a 24-h period. Cytotoxicity was assessed by measuring the intracellular LDH content [27]. The maximal concentration not causing a significant decrease in intracellular LDH, the MNTC, was determined after several assays.

2.5. Flow cytometric analysis of DNA fragmentation

Cell monolayers were kept frozen at −20 °C until the time of DNA fragmentation analysis. Then monolayers were thawed and covered with hypotonic lysis solution [28] and kept overnight at 4 °C in order to release nuclei. Propidium iodide (50 μg/ml, final concentration) was added to the nuclei suspension for fluorescent staining of DNA and nuclei suspensions were incubated for 30 min at room temperature in the dark. The degree of apoptosis was estimated from the percentage of nuclei with a DNA content lower than the diploid (2C) peak in a single-parameter histogram of PI fluorescence distribution [28].

2.6. Caspases-3, -8 and -9 activities

After incubation of hepatocytes for increasing periods of time with the different treatments, detached cells were collected by centrifugation at 500 × *g* for 3 min and attached cells were scraped off. Cells were pooled and lysed at 4 °C in a buffer (10 mM Tris–HCl, 10 mM $NaH_2PO_4/NaHPO_4$, pH 7.5, 130 mM NaCl, 1% Triton X-100, and 10 mM NaPPi), as described [28]. Caspases-3, -8 and -9 activities were measured using the specific fluorogenic substrates 20 μM Ac-DEVD-AMC, Ac-IETD-AFC and Ac-LEDHD-AFC respectively, as previously described in detail [29]. Cellular protein was determined as described [30].

2.7. Statistical analysis

Each experiment was performed on three different cultures. The statistical significance of the experimental data was analysed by the Student's *t*-test.

3. Results

3.1. Cytotoxicity of anti-Fas and FK506 on cultured hepatocytes

Cells were incubated with increasing concentrations of FK506 and CsA up to 25 μM for 24 h, and changes in intracellular LDH content were evaluated as a cytotoxicity end-point. The maximal concentration of FK506 and CsA not causing cytotoxic effect was greater than 25 μM. Cicloheximide (CHX) at 50 μg/ml did not produce any cytotoxic effect.

The effect on hepatocyte viability of anti-Fas (CH11) at concentrations up to 100 ng/ml, alone or in combination with 50 μg/ml CHX, was evaluated after 20 h of treatment to select the most effective concentration of CH11 to induce cell death. As Fig. 1 shows CH11 alone did not produce cytotoxicity to human hepatocytes, while in combination with CHX a significant reduction of hepatocyte viability was found at 25 ng/ml CH11 and at 50 ng/ml there was an 80% reduction.

3.2. Effect of FK506 on Fas-induced DNA fragmentation

Flow cytometric analysis of DNA fragmentation was performed in hepatocytes incubated for 20 h with 50 ng/ml CH11, 50 μg/ml CHX or both compounds in combination. Fig. 2 shows a significant increase of percentage of apoptotic nuclei with sub-diploid DNA content (DNA fragmentation) after Fas-induced apoptosis (ca. 90%) by CH11 combined with CHX, in comparison with unstimulated controls. The protective effect of FK506 on DNA fragmentation was evaluated. Hepatocytes were pre-treated for 3 h with 5 μM FK506 prior induction of Fas-mediated apoptosis and the percentage of sub-diploid nuclei was evaluated up to 18 h of treatment in the presence of FK506. A significant reduction (30–40%) of percentage of DNA fragmentation was found in cultures

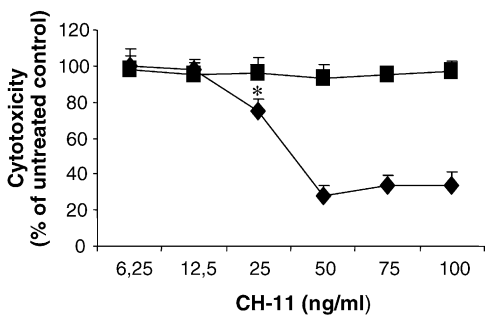

Fig. 1. Cytotoxicity of anti-FasR on cultured hepatocytes. Cytotoxicity was assessed by measuring the intracellular LDH content. The effect on hepatocyte viability of 50 ng/ml CH11 alone (■) or in combination with 50 μg/ml CHX (◆) was evaluated after 20 h of treatment. Data are expressed as percentages of the untreated controls and correspond to the means ± S.D. of triplicate dishes from a representative experiment. *$p < 0.01$, Student's *t*-test, respect to hepatocytes treated with CH11 alone.

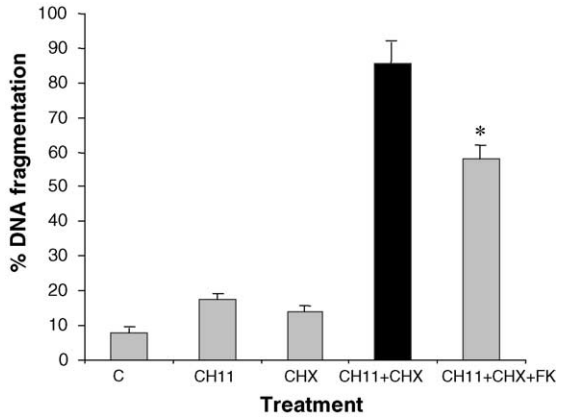

Fig. 2. Effect of FK506 on Fas-induced DNA fragmentation Fas-induced apoptotic nuclei either by 50 ng/ml CH11 or 50 μg/ml CHX or both compounds in combination was evaluated in human hepatocytes. Protective effect of FK506 on DNA fragmentation was evaluated in hepatocytes pretreated for 3 h with 5 μM FK506. Flow cytometric analysis of percentage of apoptotic nuclei with sub-diploid DNA content (DNA fragmentation) was performed in hepatocytes incubated for 20 h. Data correspond to the mean ± S.D. of triplicate dishes from a representative experiment. *$p < 0.01$, Student's *t*-test, respect to CH11 plus CHX treated hepatocytes.

exposed to FK506. The drug alone did not produce any effect on DNA fragmentation (data not shown).

3.3. Fas-induced caspase cascade activation pathway

The kinetics of activation of caspases-3, -8 and -9 were evaluated in hepatocytes treated with 50 ng/ml CH11, 50 μg/ml CHX or both compounds in combination. The results show a time-dependent increase of caspase-3 activation in hepatocytes only in cultures treated with CH11 plus CHX peaking after 9 h of treatment (Fig. 3A). Kinetics of caspase-8 and caspase-9 activation were analysed in the same cultures and the activation reached the maximum respectively after 6 and 9 h of treatment with the apoptotic inducer (Fig. 3B and C).

To analyse the apoptotic pathway involved in the caspase activation cascade by CH11 the effect of specific inhibitors of the effector caspases-8 and -9 on activation of caspase-3 was investigated. As Fig. 3D shows, the inhibition of both caspases prevented caspase-3 activation in human hepatocytes, suggesting that both effector caspases are involved in Fas-induced apoptosis. However, a different contribution of each effector caspase on caspase cas-

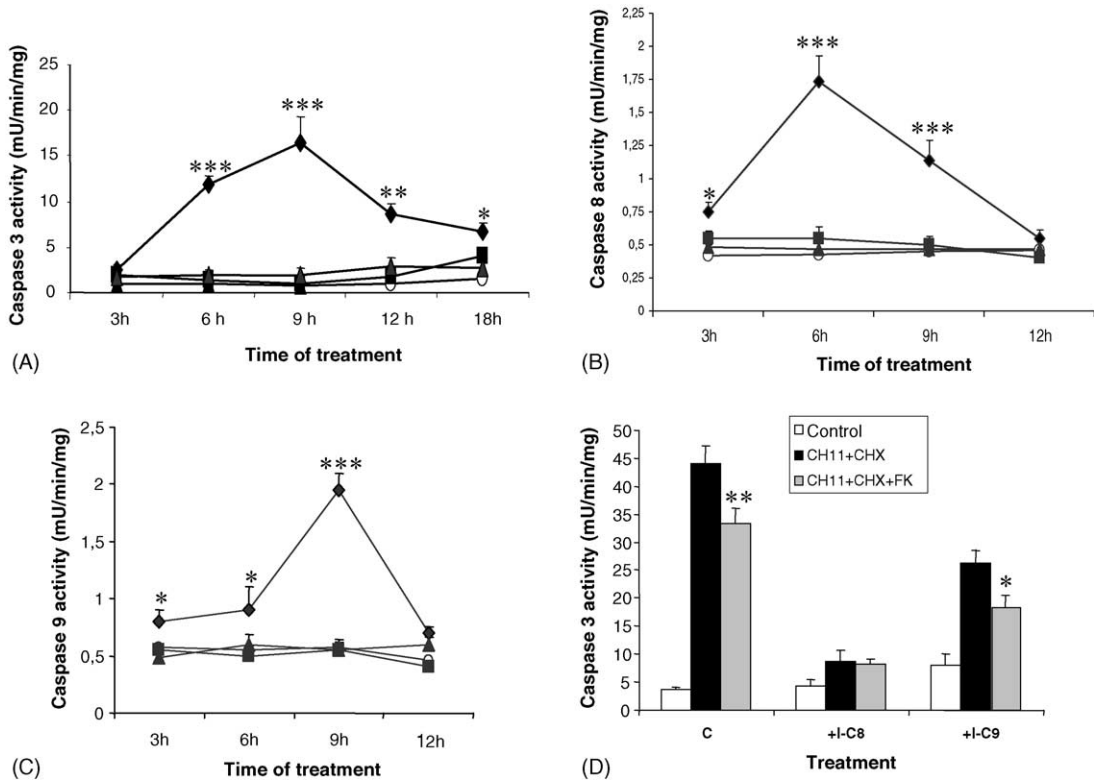

Fig. 3. Fas-induced caspases activation The kinetics of caspases-3, -8 and -9 activation was evaluated in hepatocytes treated either with 50 ng/ml CH11 (■) or 50 μg/ml CHX (▲) or both compounds in combination (◆) and compared with controls (○). Caspases-3, -8 and -9 activation was assayed by using the fluorescent substrates Ac-DEVD-AMC, Ac-IETD-AFC and Ac-LEHD-AFC, respectively. (A) A time-dependent increase in caspase-3 activation was found in hepatocytes incubated with CH11 plus CHX, reaching the maximum after 9 h of treatment. (B) The time-course of caspase-8 activation shows a maximum after 6 h of treatment with CH11 plus CHX. (C) The time-course of caspase-9 activation shows a maximum after 9 h of treatment with CH11 plus CHX. (D) To analyse the contribution of caspases to Fas-induced apoptosis, hepatocytes were exposed to 100 μM cell permeable caspase inhibitors of the caspases-8 (IETD-CHO) and -9 (LEHD-CHO) 1 h before inducing apoptosis. The role of the effector caspase inhibitors on caspase-3 activation was evaluated after 9 h of induction of apoptosis in presence and absence of FK506. Data correspond to the mean ± S.D. of triplicate dishes from a representative experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ Student's *t*-test, respect to CH11 plus CHX treated hepatocytes.

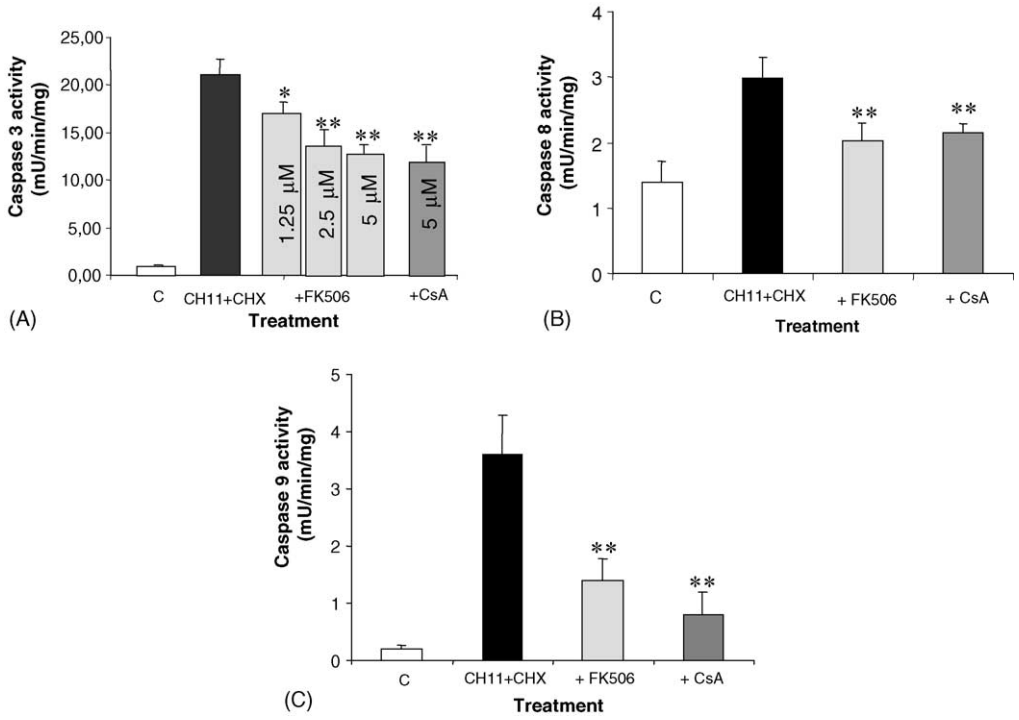

Fig. 4. Protective effect of FK506 on caspase-3 activation FK506 or CsA was added 3 h before the stimulation of apoptosis by 50 ng/ml CH11 and 50 μg/ml CHX. (A) Caspase-3 and (B) caspase-8 activation was assayed after 9 h of induction of apoptosis by using the fluorescent substrates Ac-DEVD-AMC and Ac-LEDHD-AFC, respectively, and (C) caspase-9 activation was assayed after 6 h of induction of apoptosis with Ac-IETD-AFC. Untreated control (white bars), CH11 plus CHX stimulated hepatocytes (black bars), and in Fas-induced cells pre-treated either with 5 μM FK506 (light grey bars) or with 5 μM CsA (deep grey bars). Data correspond to the mean ± S.D. of triplicate dishes from a representative experiment. *$p < 0.05$, **$p < 0.01$, by Student's *t*-test, with respect to CH11 plus CHX treated hepatocytes.

cade activation was observed. The inhibitor of the apical caspase-8 totally blocked activation of caspase-3 activation, while the caspase-9 inhibitor reduced caspase-3 activation by 35%, thus indicating a significant contribution of the mithochondrial pathway in Fas-mediated apoptosis.

Next we analysed the protective effect of FK506 and CsA on caspase-3 activation by adding the compounds 3 h before the stimulation of Fas-induced apoptosis. The results showed that both FK506 and CsA significantly reduced caspase-3 activation (40%) when human hepatocytes were pre-treated with 5 μM FK506 or CsA (Fig. 4A). A similar protective effect of FK506 on caspase-8 (Figs. 3D and 4B) and caspase-9 (Fig. 4C) activation was found. Non-significant protection was observed when the FK506 was added simultaneously to CH11 and CHX (data not shown).

4. Discussion

Apoptosis, or programmed cell death, is considered a physiological mechanism of organ homeostasis, but it could be enhanced in some diseases and pathological status, and has been related to allograft rejection and ischemia–reperfusion injury in liver transplantation [1,31]. Apoptotic signalling includes three stages: signal induction, which varies with the stimulus but converges on a common propagation stage via caspase cascade activation, and an execution stage. The caspase cascade has two main modes of activation: at the cell membrane death receptors, including FasR, and at the mitochondria involving release of mitochondrial proteins and activation of initiator caspase-9 [1,32].

FasL binding to its death receptor, FasR, results in recruitment of the receptor-specific adapter protein Fas-associated death domain (FADD), which then recruits caspase-8. Activated caspase-8 is known to propagate the apoptotic signal either by directly cleaving and activating downstream caspases or by cleaving Bid, the Bcl2-interacting protein, that translocates to mitochondria and leads to the release of cytochrome c from mitochondria, triggering activation of caspase-9 [1,32–34]. Thus, the death receptors can activate multiple death domain-initiated apoptosis programs, including both extrinsic and intrinsic pathways [34,35]. The activation of intracellular signalling leads to the activation of endonucleases resulting in DNA fragmentation that finishes with cell death.

FasR is expressed on the cell surface of normal hepatocytes [11,33], although its levels are significantly higher in acute rejection after liver transplantation and other inflammatory conditions [4]. It has been shown previously that FasR expressed in cultured mouse hepatocytes functionally transduces the apoptotic signal but at the same

time hepatocytes also express protective proteins against Fas-mediated apoptosis. Therefore, it was shown that simultaneous addition of actinomicyn D or cycloheximide to anti-FasR greatly increases Fas-dependent apoptosis [35–37]. Our results show, in agreement with these findings, that the combination of CHX with low concentrations of agonistic antibodies against FasR (CH11), which is known to induce cell death based on Fas-mediated apoptosis [11,26], appears to be necessary for decreasing the percentage of hepatocytes escaping from Fas-mediated apoptosis.

The immunomodulating agents selective T-cell suppressors like the polypeptide CsA and the macrolide FK506 have been claimed to possess hepatoprotective properties at low doses [13–15]. The role of CsA on apoptosis following warm ischemia–reperfusion [22] and mitochondria protection [22,23] has been previously well established. The main objective of our work was to analyse the preventive effect of FK506 on Fas-induced apoptosis in human hepatocytes pre-treated with the drug in comparison with CsA. The results show that pre-treatment with FK506 significantly reduced DNA fragmentation and downstream caspases-3, -8 and -9 activation in cultured hepatocytes stimulated to apoptosis via Fas (Figs. 2 and 4). These observations on human hepatocytes may help explain the low incidence of acute cellular rejection in patients treated with FK506 [14,15]. Active cell death induced by binding of FasR with its ligand, FasL, plays a major role in cell killing via apoptosis by cytotoxic T-lymphocytes [38]. Thus, T-cell injury of hepatocytes during graft rejection in liver transplantation is very likely related to Fas-mediated apoptosis [12]. Therefore, Fas-antigen expression of hepatocytes and its modification by immunosuppressive agents such as FK506 could influence allograft survival. There is a decrease in FasR expression in mouse hepatocytes cultured in the presence of FK506 [16,17] and in warm ischemia–reperfusion injury in liver of rats pre-treated with CsA [22]. These findings suggest that in the in vivo setting, hepatocytes of the allograft would have a lower chance of being attacked by cytotoxic T-lymphocytes in hosts treated with immunosuppressive drugs such as FK506 and CsA. Therefore, the modification of FasR expression of hepatocytes by FK506 could influence allograft survival.

The role of FK506 in tissue protection from ischemia–reperfusion injury may not be the consequence of a single pathway, but caused by multiple inter-related mechanisms. Clinically, anti-apoptotic effects of FK506 have been suggested that could be closely related to the protective effect of the drug on mitochondria [21]. Indeed the FK506 binding protein of the mitochondria is the receptor for this compound and plays a key role in the regulation of MTP [39]. The results clearly show the contribution of the mitochondrial pathway in Fas-mediated apoptosis (Fig. 3D), and the preventive effect of FK506 on caspase-9 activation in Fas-induced apoptosis (Fig. 4). It has been described that FK506 inhibition of the onset of MPT does not require interaction with calcineurin, indicating a dissociation between immunosuppression and mitochondrial protection [39,40]. Thus suggests a cytoprotective effect of FK506 against liver injury through a direct anti-apoptotic effect in human hepatocytes by preventing the mitochondrial dysfunction and the caspase cascade activation pathway.

In summary, a downregulatory effect of the immunosuppressive drug FK506 on FasR expression [16,17], which may lead to a decrease of active caspase-8 levels and thus, prevents apoptosis; and the results also point to a direct protective effect of FK506 on the apoptotic pathway at the intracellular level. Since Fas-mediated apoptosis relies on caspase-8 activation and mitochondria to activate caspase-3, the protection of mitochondria seems to prevent mitochondrial protein release [39,40], which in turn very likely decreases caspase-9 activation and the downstream activation of caspase cascade, thus reducing the propagation and amplification of the apoptotic signal.

Our observation of a preventive effect of FK506 against human hepatocyte apoptosis could support the use of this compound as a preconditioning agent. Studies in normothermic ischemia indicate that the initial phenomena of cell death were activated as soon as 1 h after reperfusion, with maximal caspase activation and cell death occurring within 3–6 h after reperfusion [41]. Our observation suggests that the use of this compound in a time interval before the ischemia establishment as a preconditioning agent will obtain maximal benefits on hepatocyte apoptosis following transplantation. Further studies are necessary to clarify this point.

Although the human peak concentrations are lower compared to those needed in cultured hepatocytes to prevent apoptosis, in animal studies it has been proved that FK506 widely distributed into tissues with a high accumulation in liver [42].

We conclude that the pre-treatment of cultured human hepatocytes with FK506 protects against Fas induced apoptosis and mitochondrial dysfunction with similar potency as CsA. The protective effect of FK506 against human hepatocyte apoptosis could support the clinical use of this compound as a promising preconditioning agent against ischemia–reperfusion injury.

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References

[1] Schuchmann M, Galle PR. Apoptosis in liver disease. Eur J Gastroenterol Hepatol 2001;13:785–90.

[2] Krams SM, Egawa H, Quinn MB, Villanueva JC, García-Kennedy R, Martínez OM. Apoptosis as a mechanism of cell death in liver allograft rejection. Transplantation 1995;59:621–5.

[3] Afford SC, Hubscher S, Strain AJ, Adams DH, Neuberger JM. Apoptosis in the human liver during allograft rejection and end-stage liver disease. J Pathol 1995;176:373–80.

[4] Tannapfel A, Kohlhaw K, Ebelt J, Hauss J, Liebert U, Berr F, et al. Apoptosis and the expression of Fas and Fas ligand (FasL) antigen in rejection and reinfection in liver allograft specimens. Transplantation 1999;67:1079–83.

[5] Gollackner B, Sedivy R, Rockenschaub S, et al. Increased apoptosis of hepatocytes in vascular occlusion after orthotopic liver transplantation. Transpl Int 2000;13:49–53.

[6] Cursio R, Gugenheim J, Ricci JE, et al. A caspase inhibitor fully protects against lethal normothermic liver ischemia by inhibition of liver apoptosis. FASEB J 1999;13:253–61.

[7] Yin XM, Ding WX. Death receptor activation-induced hepatocyte apoptosis and liver injury. Curr Mol Med 2003;3:491–508.

[8] Pessayre D, Mansouri A, Haouzi A, Fromenty B. Hepatotoxicity due to mitochondrial dysfunction. Cell Biol Toxicol 1999;15: 367–73.

[9] Zamzami N, Brenner C, Marzo I, Susini SA, Kroemer G. Subcellular and submitochondrial mode of action of Bcl-2 like oncoproteins. Oncogene 1998;16:2265–83.

[10] Maeda S. Mechanisms of active cell death in isolated hepatocytes. In: Berry MN, Edwards AM, editors. The hepatocyte year book. London: Kluwer Academic Publishers; 2000. p. 281–300.

[11] Watanabe-Fukunaga R, Brannan CI, Itoh N, et al. The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J Immunol 1992;148:1274–9.

[12] Rivero M, Crespo J, Mayorga M, Fábrega E, Casafont F, Pons-Romero F. Involvement of the Fas system in liver allograft rejection. Am J Gastroenterol 2002;97:1501–6.

[13] Peters DE, Fitton A, Plosker GL, Faulds D. Tacrolimus. A review of its pharmacology, and therapeutic potential in hepatic and renal transplantation. Drugs 1993;46:764–94.

[14] US Multicenter FK506 Liver Study Group. A Comparison of tacrolimus (FK506) and cyclosporine for immunosuppression in liver transplantation. N Engl J Med 1994;331:1110–5.

[15] European FK506 Multicenter Liver Study Group. Randomised trial comparing tacrolimus (FK506) and cyclosporin in prevention of liver allograft rejection. Lancet 1994;334:423–8.

[16] Yokoyama I, Hayakawa A, Hayashi S, et al. Decreased Fas antigen expression of cultured hepatocytes with FK506. Transplant Proc 1996;28:3189–90.

[17] Yokoyama I, Hayakawa A, Hayashi S, Kobayashi T, Negita M, Takagi H. Fas antigen expression of hepatocytes and its modification by immunosuppressants. Dig Dis Sci 1997;42:2471–5.

[18] García-Criado FJ, Lozano-Sanchez F, Fernandez-Regalado J, et al. Possible tacrolimus action mechanisms in its protector effects on ischemia–reperfusion injury. Transplantation 1997;66:942–3.

[19] St Peter SD, Post DJ, Rodriguez-Davalos MI, Douglas DD, Moss AA, Mulligan DC. Tacrolimus as a liver flush solution to ameliorate the effects of ischemia–reperfusion injury following liver transplantation. Liver Transpl 2003;9:144–9.

[20] Crenesse D, Laurens M, Heurteaux C, et al. Rat liver ischemia–reperfusion-induced apoptosis and necrosis are decreased by FK506 pretreatment. Eur J Pharmacol 2003;473:177–84.

[21] St Peter SD, Moss AA, Mulligan DC. Effects of tacrolimus on ischemia–reperfusion injury. Liver Transpl 2003;9:105–16.

[22] Saxton NE, Barclay JL, Clouston AD, Fawcett J, Cyclosporin. A pretreatment in a rat model of warmischaemia/reperfusion injury. J Hepatol 2002;36:241–7.

[23] Waldmeier PC, Feldtrauer JJ, Qian T, Lemasters JJ. Inhibition of the mitochondrial permeability transition by the nonimmunosuppressive cyclosporine derivative NIM811. Mol Pharmacol 2002;62:22–9.

[24] Serralta A, Donato MT, Orbis F, Castell JV, Mir J, Gómez-Lechón MJ. Functionality of cultured human hepatocytes from elective samples, cadaveric grafts and hepatectomies. Toxicol In Vitro 2003;17:769–74.

[25] Gómez-Lechón MJ, Donato T, Ponsoda X, Fabra R, Trullenque R, Castell JV. Isolation, culture and use of human hepatocytes in drug research. In: Castell JV, Gómez-Lechón MJ., editors. In vitro methods in pharmaceutical research. London: Academic Press; 1997. p. 129–53.

[26] Yonehara S, Isshii A, Yonehara M. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med 1989;169:1747–56.

[27] Ponsoda X, Jover R, Castell JV, Gómez-Lechón MJ. Measurement of intracellular LDH activity in 96-well cultures A rapid and automated assay for cytotoxicity studies. J Tiss Cult Meth 1991;13:21–4.

[28] Gómez-Lechón MJ, O'Connor E, Castell JV, Jover R. Sensitive markers identifying toxic compounds that trigger apoptosis in cultured hepatocytes. Toxicol Sci 2002;65:299–308.

[29] Herrera B, Fernández M, Alvarez AM, et al. Activation of caspases occurs downstream from radical oxygen species production, Bcl-X_L down regulation, and early cytochrome c release in apoptosis induced by transforming growth factor β in rat fetal hepatocytes. Hepatology 2001;34:548–56.

[30] Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.

[31] Kohli V, Selzner M, Madden JF, Bentley RC, Clavien PA. Endothelial cell and hepatocyte deaths occur by apoptosis after ischemia–reperfusion injury in the rat liver. Transplantation 1999;67:1099–105.

[32] Muzio M. Signalling by proteolysis: death receptors induce apoptosis. Int J Clin Lab Res 1998;28:141–7.

[33] Feldmann G, Lamboley C, Moreau A, Bringuier A. Fas-mediated apoptosis of hepatic cells. Biomed Pharmacother 1998;52:378–85.

[34] Zhao Y, Ding WX, Qian T, Watkins S, Lemasters JJ, Yin XM. Bid activates multiple mitochondrial apoptotic mechanisms in primary hepatocytes after death receptor engagement. Gastroenterology 2003;125:854–67.

[35] Feldmann G, Haouzi D, Moreau A, et al. Opening of the mitochondrial permeability transition pore causes matrix expansion and outer membrane rupture in Fas-mediated hepatic apoptosis in mice. Hepatology 2000;31:674–83.

[36] Rouquet N, Carlier K, Briand P, Wiels J, Joulin V. Multiple pathways of Fas-induced apoptosis in primary culture of hepatocytes. Biochem Biophys Res Commun 1996;229:27–35.

[37] Jones RA, Jonson VL, Buck NR, et al. Fas-mediated apoptosis in mouse hepatocytes involves the processing and activation of caspases. Hepatology 1998;27:1632–42.

[38] Kägi D, Vignaux F, Ledermann B, et al. Fas and perforin pathways as major mechanisms of T-cell-mediated cytotoxicity. Science 1994;265:528–30.

[39] Farghali H, Masek K. Immunopharmacologic agents in the ameloriation of hepatic injuries. Int J Immunopharmacol 1998;20:125–39.

[40] Kaibori M, Inoue T, Tu W, et al. FK506, but not cyclosporin A, prevents mitochondrial dysfunction during hypoxia in rat hepatocytes. Life Sci 2001;69:17–26.

[41] Cursio R, Gugenheim J, Ricci JE, et al. A caspase inhibitor fully protects against lethal normothermic liver ischemia by inhibition of liver apoptosis. FASEB J 1999;13:253–61.

[42] Christians U, Jacobsen W, Benet LZ, Lampen A. Mechanisms of clinically relevant drug interactions associated with tacrolimus. Clin Pharmacokinet 2002;41:813–51.