Current Topics in
Toxicology
Vol. 1, 2004

Evaluation of chemical induced apoptosis in cultured hepatocytes

María José Gómez-Lechón*
Unidad de Hepatología Experimental, Centro de Investigación, Hospital Universitario La Fe, Avda de Campanar 21, E-46009 Valencia, Spain

Abstract

It is generally believed that mild forms of injury induce apoptosis, while more severe forms of insult result in necrosis. Moreover, in the liver, as in other tissues, many compounds can even cause apoptosis and necrosis simultaneously. The two most well-studied pathways of apotosis include the surface death receptor pathway (i.e. Fas) and the mitochondria-initiated pathway. Mitochondria are deeply involved in the regulation of cell death, undergoing membrane permeabilization which commits hepatocytes to apoptosis. Apoptosis may be a major event in chemical-induced injury and therefore the detection of apoptotic effects when developing new drugs is highly relevant in screening for pharmaco-toxicologic risk assessment. However, as apoptosis *in vitro* normally degenerates to secondary necrosis, it may be underestimated unless sensitive and early parameters of apoptosis are used. The markers selected include several biochemical parameters (down-regulation of the anti-apoptotic $bclX_L$ gene, caspase activation and cytochrome c release from mitochondria), and flow cytometry determinations (chromatin complexity and DNA integrity). The apoptotic effect can be generally detected at low concentrations of the drugs long before cell necrosis, but some compounds induce apoptosis concomitantly to necrosis. Among the markers evaluated caspase 3 activation, and nuclei and DNA analysis by flow cytometry fulfil the compromise between reliability, sensitivity and ease of performance.

Key words: apoptosis, caspases, cytotoxicity, diclofenac, drugs, necrosis

*e-mail: gomez_mjo@gva.es

Introduction

For many years it was assumed that chemically induced injury and death occurred primarily by necrosis. Now, however, it is recognized that cell death may also be the result of another mechanism, namely apoptosis, which could be induced by the absence of survival signals or activation of death receptors by different lethal signals [1]. A better understanding of the fundamental mechanisms involved in apoptosis has made it possible to define its real significance in many areas of cell biology and more recently in pharmacology and toxicology. In fact, it is now believed that apoptosis could be the major form of chemically induced cell death and that necrosis is much rarer, occurring only in circumstances of gross cell injury [1-3]. However, the importance of apoptosis in toxicology has been underestimated because of the difficulty in identifying apoptotic cells in the intact organism, because they undergo striking morphological changes, that make them swiftly recognizable and rapidly engulfed by phagocytes [1,3, 4]. During *in vitro* experimentation, in the absence of phagocytes, a secondary non-specific degeneration occurs which results in the uptake of vital dyes such as trypan blue which is commonly mistaken for necrosis and is often referred to as secondary necrosis. Therefore, apoptotic cells may be underestimated, particularly *in vitro,* unless specific and sensitive parameters are used. The biochemical stages of apoptosis induced by a toxicant can be described as the imposition of damage by the toxicant, sensing, coupling the damage to the engagement of apoptosis, and execution of the cell and disposal of the corpse [3]. In this context, apoptosis provides a mechanism for the disposal of cells damaged by toxicants without perturbing the homeostatic balance of its environment. It has been investigated whether it would be possible to

identify compounds that induce the basic mechanisms of apoptosis in hepatocytes, when very mild or non necrotic effects are observed, as well as to determine the appropriate apoptotic markers able to predict the apoptotic potential of compounds.

Apoptotic pathways

Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Upon receiving specific signals instructing the cells to undergo apoptosis a number of distinctive biochemical and morphological changes occur in the cell. A family of proteins known as caspases are typically activated in the early stages of apoptosis. These proteins breakdown or cleave key cellular substrates that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus. The result of these biochemical changes is appearance of morphological changes in the cell.

There are a number of mechanisms through which apoptosis can be induced in cells. In some cases the apoptotic stimuli comprise extrinsic signals such as the binding of death inducing ligands to cell surface receptors [5] (Figure 1). In other cases apoptosis is initiated following intrinsic signals that are produced following cellular stress. Cellular stress may occur from exposure to radiation or chemicals or to viral infection. In general intrinsic signals initiate apoptosis via the involvement of the mitochondria. [6-8].

Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific ligands. They play an important role in apoptosis and can activate a caspase cascade within seconds of ligand binding. Induction of apoptosis via this mechanism is therefore very rapid. Death receptors belong to the tumour necrosis factor (TNF) gene superfamily and generally can have several functions other than initiating apoptosis. The best characterised of the death receptors are CD95 (or Fas), TNFR1 (TNF receptor-1) and the TRAIL, (TNF-related apoptosis inducing ligand) receptors DR4 and DR5 [9]. Induction of apoptosis via death receptors results in the activation of an initiator caspase such as caspase 8 or caspase 10, which in turn cleave Bid, a recently described bcl-2 family protein, to a truncated form that translocates to mitochondria and initiates the mitochondrial apoptotic pathway. These caspases activate other caspases in a cascade. This cascade eventually leads to the activation of the effector caspases, such as caspase 3 and caspase 6 which are responsible for the cleavage of the key cellular proteins that leads to the typical morphological changes observed in cells undergoing apoptosis.

Mitochondria are deeply involved in the regulation of cell death, undergoing membrane permeabilization and release of several mitochondrial proteins into cytoplasm which commits hepatocytes to apoptosis (Figure 1). The bcl-2 proteins are a family of proteins involved in the response to apoptosis. Some of these proteins (such as bcl-2 and bcl-X_L) are anti-apoptotic, while others (such as Bad, Bid or Bax) are pro-apoptotic. The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic bcl-2 proteins. When there is an excess of pro-apoptotic proteins the cells are more sensitive to apoptosis, when there is an excess of anti-apoptotic proteins the cells will tend to be less sensitive. For example, anti-apoptotic members of the bcl-2 family of proteins are located in the outer mitochondrial membrane and act to promote cell survival. Many of the pro-apoptotic members of the bcl-2 family, such as bax, bak and bid, also mediate their effects though the mitochondria, either by direct interactions with the mitochondrial membrane [10] or by interacting with Bcl-2 and Bcl-X_L, the only member of the bcl-2 family present in hepatocytes [6, 11]. The pro-apoptotic bcl-2 proteins are often found in the cytosol where the act as sensors of cellular damage or stress [10, 12]. Following cellular stress they relocate to the surface of the mitochondria where the anti-apoptotic proteins are located. This interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic bcl-2 proteins and can lead to the formation of pores in the mitochondria and the release of cytochrome c from the intermembrane space, which together with Apaf-1 and ATP forms a complex with pro-caspase 9, so called apoptosome, leading to activation of caspase 9 and the caspase cascade (Figure 1). It is unknown what factors promote the release of cytochrome c from the mitochondria. Since Bax, and other Bcl-2 proteins, show structural similarities with

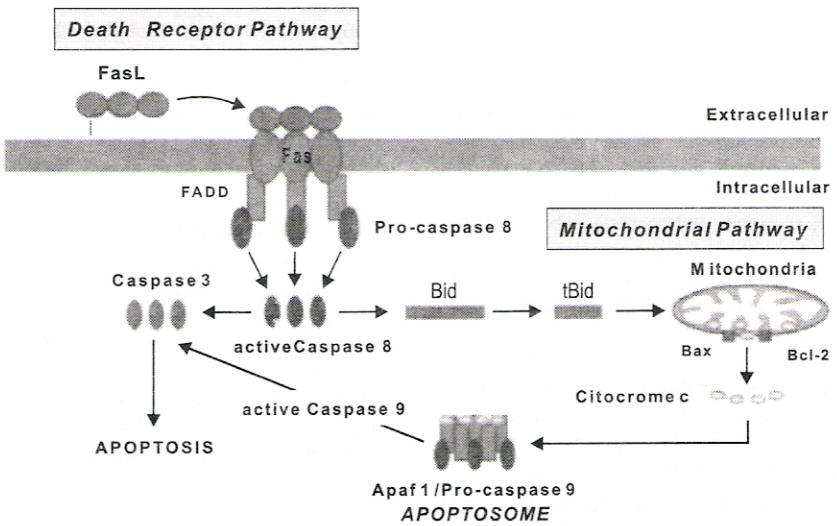

Figure 1. Apototic pathways.

The two most well-studied pathways of apotosis include the surface death receptor pathway, that recruits the effector caspase 8 and mediates transduction of the death signal in cells and the mitochondria-initiated pathway. Mitochondria are deeply involved in the regulation of cell death, undergoing membrane permeabilization and release of several mitochondrial proteins into cytoplasm which commits hepatocytes to apoptosis

pore-forming proteins. It has therefore been suggested that Bax can form a transmembrane pore across the outer mitochondrial membrane, leading to loss of membrane potential and efflux of cytochrome c and AIF (apoptosis inducing factor). It is thought that Bcl-2 and Bcl-X_L act to prevent this pore formation. Heterodimerisation of Bax or Bad with Bcl-2 or Bcl-X_L is thought to inhibit their protective effects. It is also thought that proteins such as Bax and Bad can promote the formation of the large diameter pore, with subsequent loss of cytochrome c and initiation of apoptosis. This constitute the execution phase of apoptosis, and it appears to be the point-of-no-return of apoptosis [12]. In this context, the decreased expression of bclX_L and the release of cytochrome c are very specific biomarkers for the mitochondrial pathway of apoptosis [3, 7].

The caspases are a group of cysteine proteases that exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis. The caspases are the most important effector molecules that play a central role in the execution of apoptosis [13]. Progression of the caspase activation cascade ends with the activation of caspase 3, that occurs in the early apoptosis, long before DNA-fragmentation appears. Once caspase 3 has been activated, there is no way back to normal viability; the program for cell death is irreversibly activated. Therefore, it is considered a very specific and sensitive apoptotic marker irrespective of how cell death is initiated, [13].

Chemical induced apoptosis to hepatocytes.

The liver is the most active organ in metabolizing foreign compounds, and although biotransformation catalyzed by cytochrome P450 enzymes generally parallels a detoxification process, this reaction can generate metabolites that are not only more reactive but also more toxic than the parent compound. Therefore the hepatic metabolism of drugs is very frequently the

cause of adverse drug reactions in the liver and the reason why the liver is a key target organ for drug toxicity [14, 15]. Low molecular weight organic chemicals can be transformed by normal drug-metabolising systems into short-lived metabolites that are inherently reactive towards cellular macromolecules. There is direct evidence that the formation of such chemically reactive metabolites may lead to mutagenesis, carcinogenicity, apoptosis and necrosis in both cell and animal models. A number of drugs associated with non-pharmacological drug toxicities in man have been shown to undergo bioactivation either *in vivo* or *in vitro*. Since it is now recognized that apoptosis in the liver plays a central role in the toxicity of many xenobiotics and cytochrome P450-generated metabolites, the early detection of the apoptotic potential to hepatocytes of new pharmaceuticals under development is of great pharmaco-toxicological relevance [11, 16, 17].

The identification of apoptotic drugs remains one of the highest priorities of the pharmaceutical industry. Most drugs in development for cancer will at some time in their development be tested for apoptotic activity and indeed screening of proapoptotic molecules remains a strategy for identifying new cancer therapies. On the other hand, the inhibition of apoptosis is an option for the treatment of various ischemic diseases such as myocardial infarction and neuro-degeneration. In order to identify both pro- and anti-apoptotic molecules, reproducible, easy-to-handle, and rapid microplate-based assays that are amenable to high throughput screening (HTS) are required. It has been proposed that drug-induced apoptosis is mainly mediated by the mitochondrial pathway, which involves mitochondrial permeabilty transition (MPT), a non-selective inner membrane permeabilization, considered as a common mechanism that may precede necrotic and apoptotic cell death [5, 7]. Therefore, the interest interest focus on assays developed to determine apoptotic events within the mitochondrion, in particular the mitochondrial permeability transition event which is implicated in the activation phase of apoptosis and necrosis.

Although two main pathways of apotosis have been described the diverse molecules (chemicals and death receptor ligands) may differentially alter these apoptotic mechanisms. Several examples can be found in the literature. For example induction of cytochrome c release in absence of caspase 3 activation by etoposide in human leukemia cells has been reported [18], as having been Fas-induced apoptosis by multiple pathways [19]. Apoptotic cell death through both caspase-dependent and caspase-independent pathways in primary culture of hepatocytes has also been described [20]. More recently aspirin has been shown to trigger cytochrome c release preceeding caspase activation and loss of mitochondrial membrane potential [10].

Evaluation of apoptosis.

Apoptotic markers associated with the pivotal steps of the execution phase should be evaluated to accurately identify compounds able to induce apoptosis after mild injury. It has been proposed a selection of apoptotic markers aimed at identifying apoptotic compounds irrespective of the pathway of how cell death was initiated and trying to find a compromise between specificity, sensitivity and ease of detection. Some markers involve the use of intact cells (flow cytometric analysis of nuclei) and others the evaluation of biochemical parameters in subcellular fractions (cytochrome c release, $bclX_L$ gene expression, and caspase 3 activation [16].

Several assays have been described involving the use of DNA intercaling dyes in order to estimate the number of apoptotic cells in culture (propidium iodide, acridine orange, Hoechscht 33258), but flow cytometry allows a much more accurate automatized analysis. Other assays can be applied in living cultured cells: a) the use of annexin V allows the detection of phosphatidyl serine, which is restricted to the inner surface of the plasma membrane, but it is externalized during apoptosis triggering phagocytosis *in vivo* [21, 22], and b) the poly (adenosine diphosphate-ribose) polymerase (PARP) is cleaved by activated caspase-3 leading to apoptosis [13]. Methods to evaluate DNA fragmentation and localization of DNA strand breaks, as a result of the activation of endonucleaes, in tissue sections and in cultured cells have been also developed. TUNEL assay is considered a standard assay for apoptosis, both in flow- and image cytometry. TUNEL assay is used to detect the DNA strand breaks which are a hallmark of the late stages of apoptosis, leading to DNA fragmentation and loss of low-molecular, internucleosomal DNA fragments which may be revealed by DNA gel electrophoresis (the "DNA-ladder" assay [13]. However, these assays provide information overlaping other apoptotic markers and do not fully fit for the requirements for throughput screening purposes: easy to perform, little time- and

sample-consuming, automatizable and relatively unexpensive.

The selection of apoptotic markers should be made on the basis of 1) to be able to identify apoptotic compounds irrespectively of the pathway of how cell death was initiated, 2) to find a compromise between specificity, sensitivity, ease performance and reproducibility, 3) a need of a small amount of cells, low cost methods, and 4) automatized analysis whenever possible. In this regard, it has been suggested that the combined use of caspase 3 activation, and nuclei and DNA analysis by flow cytometry fulfil the requirements for screening the apoptotic effect of new drugs on hepatocytes [16]

Evaluation of chemical-induced apoptosis.

Apoptotic effects as a consequence of a mild injury were analyzed after incubating hepatocytes with concentrations of model drugs (D-galactosamine, curcumin, campothecine, etoposide and t-butyl-hydroperoxide) without causing observable intracellular LDH-leakage, therefore indicating the integrity of the outer cell membrane [16].

It was analysed the apoptotic effect of topoisomerase-I and II inhibitors, campothecine and etoposide respectively, known to produce apoptosis both in primary cells and immortalized cell lines [23, 24]. Our results show that long before LDH-leakage all the apoptotic markers evaluated became altered, which means that both curcumine and topoisomerase inhibitors are potent apoptotic inducers in hepatocytes (Figure 2A and B and Figure 3). Curcumin, the active ingredient of the rhizome of Curcuma longa, has antiproliferative and antitumoral effects on several cell

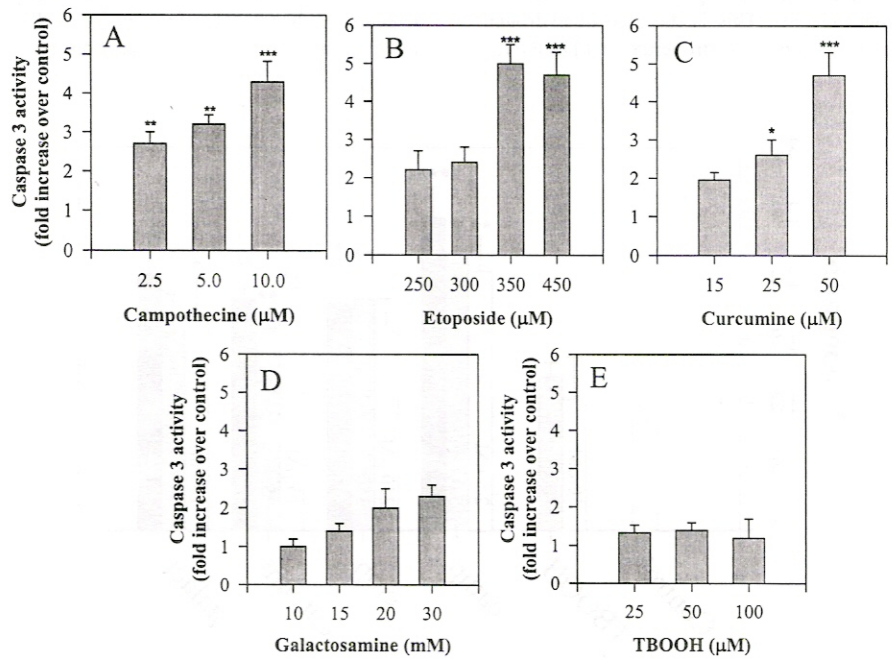

Figure 2. Dose-dependent effect of compounds on caspase 3 activation.

Cultured hepatocytes were exposed to increasing sub-cytotoxic concentrations of the compounds, for an 8-hour period, and caspase 3 activity was measured at the end of the treatment. Effects of campothecine (A), etoposide (B), curcumin (C), galactosamine (D) and TBOOH (E). Data are expressed as the mean ± SD of three different experiments.

types both *in vivo* and *in vitro* [25-27], and it is a potent inhibitor of transcriptional factors involved in cell survival [27]. Previous reports have shown its ability to induce apoptosis in osteoclasts and AK-5 tumor cell line by producing reactive oxygen species, loss of mitochondrial membrane potential and DNA fragmentation [25, 27]. Both DNA fragmentation and caspase 3 activation were induced dose-dependently by curcumine to hepatocytes at sub-cytotoxic concentrations (Figure 2C and Figure 3). High doses of D-galactosamine have been reported to induce apoptosis in hepatocytes both *in vitro* and *in vivo* before causing necrosis [28, 29]. Increased DNA fragmentation but almost no association with caspase 3 activation, has also been reported [16, 30]. These previous findings are consistent with the results obtained in our study (Figure 2D and Figure 3) [16].

Many toxins induce apoptosis by producing active oxidants [31, 32]. This is the case of hydrogen peroxides, such as t-butyl-hydroperoxide (TBOOH), which have been described as triggering-off apoptosis in cultured hepatocytes [33-35]. It has been reported that in oxidative stress to hepatocytes induced by TBOOH, NAD(P)H oxidation, increased mitochondrial Ca^{2+}, and mitochondrial generation of reactive oxygen species precede and contribute to the mitochondria onset of the MTP. Progression to necrosis or apoptotis depends on the effect the MTP has on cellular ATP levels. If ATP levels fall sharply, necrotic killing ensues, but if the levels are partially maintained, apoptosis follows the MTP [36]. Exposure of hepatocytes to sub-cytotoxic concentrations of TBOOH produced a very moderate alteration of the apoptotic markers evaluated here, and only, when necrotic process was observed with cytotoxic concentrations, the apoptotic parameters were altered concomitantly (Figures 2E and Figure 3).

These results demonstrated that apoptosis can be detected, in hepatocytes long before cell necrosis, by low concentrations of the drugs. Four out of five well-

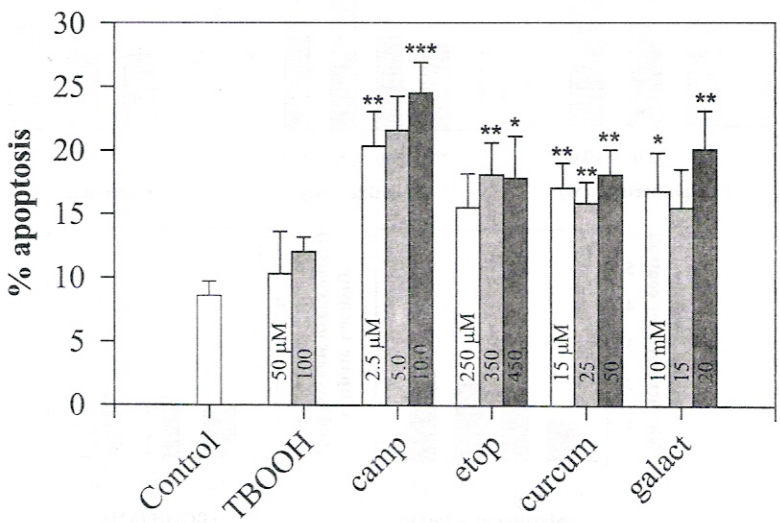

Figure 3. Effect of the different treatments on the appearance of apoptotic nuclei with sub-diploid DNA content.

The degree of apoptosis was estimated from the percentage of nuclei with DNA content lower than the 2C peak as a result of loss of nuclear DNA integrity in a single-parameter histogram of PI fluorescence distribution. Dose-dependent effect evaluated after 12-hour incubation with increasing concentrations of the drugs. Data are expressed as the mean ± SD of three different experiments.

known apoptotic compounds could be predicted at sub-cytotoxic concentrations with this battery of markers. However, for a reliable screening of apoptosis in hepatocytes several key markers should be used simultaneously [16].

Among the methods using intact cells, flow cytometry is probably the most efficient [37]. Loss of DNA integrity following endonuclease activation leads to a population of sub-diploid cells or nuclei observable when stained with a DNA-specific fluorochrome [38]. Cytometric analysis showed that most of the drugs tested increased the percentage of sub-diploid DNA content a well established marker of a late phase of apoptosis. Previous flow cytometric analysis showed that DNA stainability is affected by chromatin conformation [39]. The alterations in chromatin conformation at this early stage of culture injury may affect the accessibility of fluorochromes to DNA, thus changing the fluorescence properties of stained nuclei. Heterogeneity of DNA staining in isolated nuclei might also arise from the presence of individual nuclei with decreased DNA content due to DNA strand breaks and subsequent loss of low molecular-weight of DNA fragments during the incubation of isolated nuclei in an aqueous buffer [40]. Therefore, these results are consistent with the presence of a percentage of nuclei with early signs of apoptosis caused by xenobiotics, which contributes to the variability of DNA staining of cells belonging to the same ploidy class. Thus, although the mechanism of the increased heterogeneity can be attributed to changes in chromatin conformation, DNA strand breaks or both. Our results suggest that the combination of morphological and DNA-dependent flow cytometric parameters that are relatively simple to determine may be of interest in identifying apoptotic drugs.

Drug-induced apoptosis: Diclofenac as an example.

Diclofenac, an arylacetic non-steroidal anti-inflammatory drug (NSAID), is frequently prescribed in treating rheumatic diseases and as an analgesic [41]. The use of diclofenac has been associated with occasional hepatic toxicity [42]. Early studies in animals and man [43] showed that diclofenac undergoes an extensive hepatic metabolism involving aromatic hydroxylations and conjugations. It has been recently described how cytochrome P-450-mediated metabolic activation of the drug and the formation of reactive metabolite(s) by drug oxidation is related to diclofenac hepatotoxicity [14, 44]. It was also shown that hepatocyte injury was preceded by a decrease in ATP levels, indicating that the inability of mitochondria to produce ATP is the cause of drug toxicity [14, 45]. In addition, the key role of MPT and the decrease of ATP in the pathogenesis of diclofenac-induced hepatocyte injury have been recently reported [46]. Since MPT is considered a major common mechanism for drug-induced hepatocyte necrosis and apoptosis [37], it is very likely that apoptosis is involved in the adverse effect of diclofenac. Evidence showing that NSAIDs cause apoptosis in several cell lines have been recently reported [17]. Moreover, diclofenac has been described to induce DNA fragmentation in cultured gastric mucosa cells which were inhibited by caspase inhibitors [17]. On the basis of these previous findings, the investigation of the potential apoptotic effect of diclofenac on liver cells is of great pharmacological relevance.

It was first showed by flow cytometry a time and concentration-dependent increase of apoptotic nuclei with sub-diploid DNA content after exposure to diclofenac (Figure 4A). In addition, a clear time- and concentration-dependent caspase 3 activation was produced by diclofenac (Figure 4B). Both caspases 8 and 9 are fully involved in the pathway of diclofenac-induced apoptosis, since activation of caspase 3 was totally blocked by the inhibitors of both caspases (Figure 5).

Inhibiting the MPT ameliorates caspase activation and apoptosis in several cellular systems [81]. According to this, activation of caspases 3, 8 and 9 is totally inhibited by specific blockers of MPT [46], which confirms that diclofenac-induced apoptosis is MPT dependent both in human and rat hepatocytes [17], (Figure 6A). Simultaneous incubation of diclofenac with antioxidants largely prevented caspase cascade activation and apoptosis, suggesting that oxidative stress mediates the MPT and is a crucial event in diclofenac-induced apoptosis to hepatocytes, as has been recently proposed [28,45]. This hypothesis was confirmed by the early and significant increase of reactive oxygen species (ROS) observed in hepatocytes treated with diclofenac [17], (Figure 6B). The recruitment and cleavage of pro-caspase 8 to produce the active form of caspase 8, is a critical event in death receptor-mediated apoptosis. In turn it cleaves Bid, a bcl-2 family protein, to a truncated form that

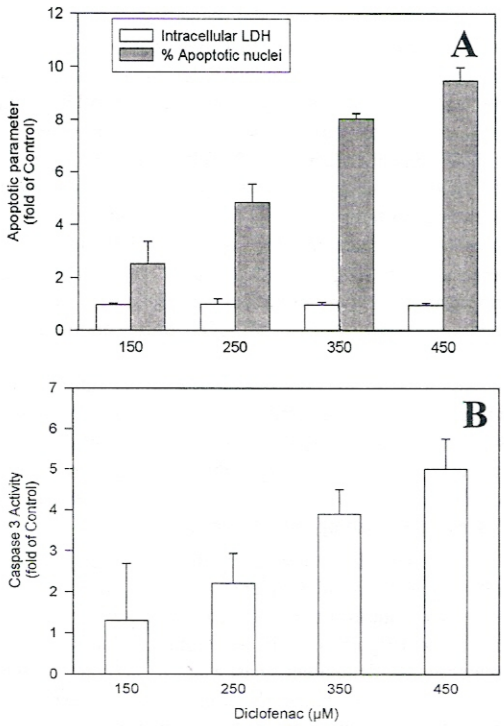

Figure 4. Flow cytometric analysis of apoptosis and caspase cascade activation by diclofenac.

Intracellular LDH release was evaluated as a cytotoxicity end-point. The degree of apoptosis was estimated from the percentage of nuclei with DNA content lower than the 2C peak in rat hepatocytes. (A) The dose-dependence of the percentage of appearance of apoptotic nuclei evaluated after 24 hours exposure to diclofenac. (B) Concentration-dependent activation of caspase 3 by diclofenac. Hepatocytes were treated for 12 hours to increasing concentrations of the drug not overlapping necrosis Data are expressed as fold increase the control values (hepatocytes not exposed to diclofenac), and represent the mean ± SD of triplicate dishes from a representative experiment.

translocates to mitochondria and initiates mitochondrial apoptotic pathway. However, Bid cleavage after treatment with diclofenac was not found [17], thus indicating that caspase 8 can be processed by diclofenac independently of cell death receptors, as has been recently

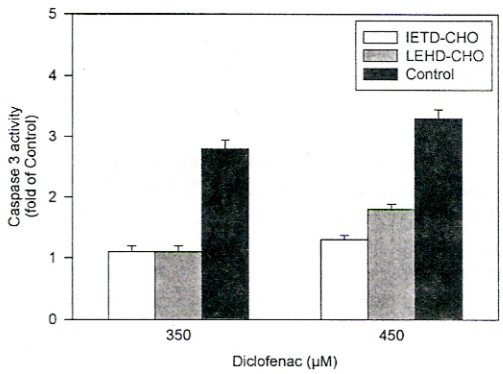

Figure 5. Effect of inhibitors of effector caspases on the caspase cascade activation by diclofenac.

Hepatocytes were exposed simultaneously to diclofenac in the presence of 100 μM cell permeable caspase inhibitors of the caspases 8 (IETD-CHO) and 9 (LEHD-CHO). The role of the effector caspase inhibitors on caspase 3 activation was evaluated after 12 hours of treatment. Data are expressed as fold increases over the control values (hepatocytes not exposed to diclofenac) and correspond to the mean ± SD of triplicate dishes from a representative experiment.

reported for anticancer drugs [49]. Since caspase 8 activation was inhibited by MPT blockers, its mitochondrial origin was, therefore, investigated. A diclofenac concentration-dependent decrease of the intra-mitochondrial levels of pro-caspase 8 was observed [17]. This finding is supported by previous reports showing pro-caspase 8 predominantly co-localized with cytochrome c in mitochondria which is released upon apoptotic stimulation through a MPT-sensitive mechanism.

The proposed mechanism for apoptosis induced by diclofenac can be summarized as follows [17]: Generation ROS would increase as a consequence of the oxidative metabolism of diclofenac, very likely acting as an early signal triggering apoptosis and activating endogenous NF-KappaB. MPT and mitochondrial dysfunction seems to be a direct and immediate consequence that would lead to a release of pro-caspase 8 and cytochrome c from mitochondria, which would contribute to the activation of caspase

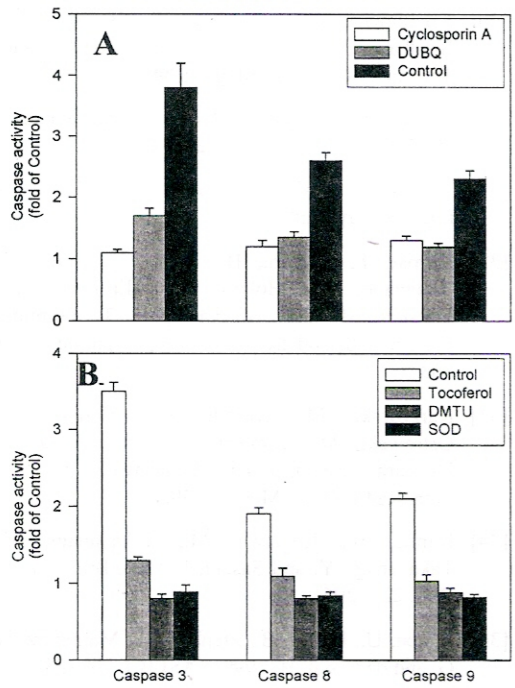

Figure 6. Effects of diclofenac at the mitochondrial level.

The involvement of the MPT in diclofenac-induced apoptosis was investigated by evaluating the effect of 5 μM Cs A and 5 μM DUBQ, MPT specific inhibitors, on caspase cascade activation after 12 hours of treatment. (B) Effects of antioxidants in diclofenac-induced MPT and prevention of caspases 3, 8 and 9 activation by in response to diclofenac. Hepatocytes were pre-treated with antioxidants DMTU, α–tocopherol, and SOD, before exposure to the drug and activation of caspases was evaluated after incubation with rat hepatocytes. Data are expressed as fold increases over the control values (hepatocytes not exposed to diclofenac), and represent the mean ± SD of triplicate dishes from a representative experiment.

9/caspase 3. Cytochrome c release seems to be caspase-independent and not mediated by activation of Bid. Caspase 3 could be responsible for processing other caspases including caspase 8.

The link between drug metabolism and toxicity to hepatocytes has been clearly established and the cytochrome P-450 isozymes involved in the production of each metabolite in man have been identified [14]. In conclusion, the results indicated that diclofenac induces apoptosis at concentrations not overlapping cell necrosis, this is related to cytochrome P-450-mediated metabolism. Oxidative injury at the mitochondrial level is involved in MPT induction, which allows the release of mitochondrial proteins, which in turn activate caspase 9 and caspase 3.

References

[1] Raffray, M. and Cohen, G. M. 1997, Pharmacol. Ther., 75, 153.

[2] Alison, M. R. and Sarraf, C. E. 1995, Hum. Exp. Toxicol., 14, 234.

[3] Gill, J. H. and Dive. C. 2000, Apoptosis in Toxicology, R. Roberts (Ed.), Taylor & Francis, New York, 1.

[4] Savill, J., Fadok, V., Henson, P. and Haslett, C. 1993, Immunology Today, 14, 131.

[5] Maeda, S. 2000, The Hepatocyte Year Book, MN Berry and AM Edwards (Eds). Kluwer Academic Publishers, London, 281.

[6] Kroemer, G.. Zamzami. N.. and Susin, S. A. 1997, Immunology Today, 18, 44.

[7] Zamzami, N., Brenner, C., Marzo, I., Susini, S. A. and Kroemer. G. 1998, Oncogene, 16, 2265.

[8] Pessayre, D., Mansouri, A.. Haouzi, A. and Fromenty, B. 1999, Cel.l Biol. Toxicol,. 15,367.

[9] Zhang, H.G., Wang,J., Yan, X., Hsu, H.C, Mountz, J.D. 2004, Oncogene, 23,2009.

[10] Piqué, M., Barragan, M., Dalmau, M., Bellosillo, B., Pons, G. and Gil, J. 2000, FEBS lett.,480,193..

[11] Feldmann, G. 1997, J. Hepatol,. 26, 1.

[12] Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P. and Wang, X. 1997, Science, 275, 1129.

[13] Cain, K. 2000). Apoptosis in Toxicology, R. Roberts (Ed.), Taylor & Francis, New York, 22,

[14] Bort, R., Ponsoda, X., Jover, R., Gómez-Lechón, M. J. and Castell, J.V. 1999, J Pharmacol. Exp. The.,. 288,65.

[15] Williams, D.P., Kitteringham, N.R., Naisbitt, D.J., Pirmohamed, M., Smith, D.A.and Park, B.K. Curr Drug Metab. 2002, 3,351.

[16] Gómez-Lechón, M. J., O´Connor, E., Castell J. V. and Jover R. 2002, Toxicol Sci. 65,299.

[17] Gómez-Lechón, M. J., Ponsoda, X., O´Connor, E. Jover, R., Donato T. and J. V. Castell. 2003, Biochem Pharmacol 66, 2155.

[18] Hirpara, J. L., Seyed, M. A., Loh, K. W., Dong, H., Kini, R. M., and Pervaiz, S. 2000, Blood 95, 1773-1780.

[19] Rouquet, N., Carlier, K., Briand, P., Wiels, J. and Joulin, V. 1996, Biochem. Biophys. Res. Commun. 229, 27.

[20] Jones, B.E., Lo, C.R., Liu, H., Srinivasan, A., Streetz, K., Valentino, K.L,, Czaja, M.J. 2000, J. Biol.. Chem. 275,705.

[21] Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L. and Henson, P. 1992, J. Immunol. 148, 2207.

[22] Ernst, J.D., Yang, L., Rosales, J. L., and Broaddus, V. C. 1998, Anal. Biochem. 260,18.

[23] Jarvis, W. D., Johnson, C.R., Fornari, F.A, Park, J. S, Dent, P. and Grant, S. 1999, .J. Pharmacol. Exp. Ther. 290, 1384.

[24] Ferraro, X. C, Quemeneur, L., Fournel, S., Prigent, A. F, Revillard, J.P. and Bonnefoy-Berard, N. 2000, Cell. Death. Differ. 7,197.

[25] Bhaumik, S., Anjum, R., Rangaraj, N., Pardhasaradhi, B. V. and Khar, A. 1999, FEBS Lett. 6, 311.

[26] Chen, H., Zhang, Z.S., Zhang, Y. L. and Zhou, D.Y. 1999, Anticancer Res. 19, 3675.

[27] Ozaki, K., Kawata, Y., Amano, S. and Hanazawa, S. 2000, Biochem. Pharmacol. 59,1577.

[28] Tsutsui, S., Hirasawa, K., Takeda, M., Itagachi, S. I., Kawamura, S., Maeda, F., Mikami, T. and Doi, K. 1997, Exp. Toxic. Pathol. 49, 301.

[29] Stachlewitz, R.F., Seabra, V., Bradford, B., Bradham, C.A., Rusyn, I., Germolec, D. and Thurman, R.G. Hepatology. 1999, 29, 737.

[30] Itokazu, Y., Segawa, Y., Inoue, N. and Omata, T. 1999, Biol. Pharm. Bull. 22, 1127.

[31] Buttke, T. M. and Sandstrom, P. 1994, Immunology. Today 25,7.

[32] Kurose, I., Higuchi, H., Miura, S., Saito, H., Watanabe, N., Hokari, R., Hirokawa, M., Takaishi, M., Zeki, S., Nakamura, T., Ebinuma, H., Kato, S. and Ishii, H. 1997, Hepatology. 25, 368.

[33] Karbowski, M,. Kurono, C., Woniak, M., Ostrowski, M., Teranishi, M., Nishizawa, Y., Usukura, J., Soji, T. and Wakabayashi, T. 1999, Free. Radic. Biol. Med. 26, 396.

[34] Kanno, S., Ishikawa, M., Takayanagi, M., Takayanagi, Y. and Sasaki, I. 2000, Biol. Pharm. Bull. 22: 1296.

[35] Rauen, U., Polzar, B., Stephan, H., Mannherz, H. G. and de Groot, H. 1999, FASEB J. 13, 155.

[36] Lemasters, J. J., Qian, T., Bradham, C. A., Brenner, C. A., Cascio, W. E. Trost, L. C., Nishimura, Y., Nieminen. A. L. and Herman, B. 1999, J. Bioenerg. Biomembr. 31, 305.

[37] Vermes, I., Haanen, C. and Reutelingsperger, C. 2000, J. Immunol. Meth. 243, 167.

[38] Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T., Traganos, F. 1997, Cytometry 27,1.

[39] Mazzini, G., Giordano, P., Riccardi, A., Montecucco, C. M. 1983, Cytometry 3, 443.

[40] Maier, P. and Schawalder, H. P. 1986, Mutation Res. 164, 369.

[41] Scully, L..J, Clarke, D. and Barr, R.J. 1993, Dig Dis Sci 38,744.

[42] Helfgott, S.M., Sandberg-Cook, J., Zakim, D. and Nestler, J. 1990, JAMA 264, 2660.

[43] Riess, W., Stierlin, H., Degen, P., Faigle, J. W., Gérardin, A., Moppert, J., Sallmann, A. et al. 1978, Scand J Rheumatol 22:17-29.

[44] Miyamoto, G., Zahid, N. and Uetrecht, J.P. 1997, Chem Res Toxicol. 10,414.

[45] Ponsoda X, Bort R, Jover R, Gómez-Lechón MJ, Castell JV. 1995, Toxic. in Vitro 9,439.

[46] Masubuchi, Y., Nakayama, S. and Horie, T. 2002, Hepatology 35,544.

[47] Waldmeier, P.C., Feldtrauer, J.J., Qian, T. and Lemasters, J.J. 2002, Mol Pharmacol 62,22.